Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/011297

International filing date: 12 April 2004 (12.04.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/462,554
Filing date: 11 April 2003 (11.04.2003)

Date of receipt at the International Bureau: 30 August 2004 (30.08.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1214690

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 19, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/462,554*
FILING DATE: *April 11, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/11297*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

04-15-03 60462554 04/11/03 Approved

PTO/SB/16 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No. EL 885032051 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jack	Taunton	San Francisco, CA
Michael	Cohen	San Francisco, CA

☒ Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

SELECTIVE SERINE/THREONINE KINASE INHIBITORS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☒ Customer Number 20350 → *Place Customer Number Bar Code Label here*

OR *Type Customer Number here*

☐ Firm or Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification *Number of Pages* 37 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 25 ☒ Other (specify) Title Page (1 pg.); return postcard

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

		FILING FEE AMOUNT ($)
☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:	20-1430	80
☐ Payment by credit card. Form PTO-2038 is attached.		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: **National Institutes of Health A144009**

Respectfully submitted,

SIGNATURE _____ Date 4/11/03

TYPED or PRINTED NAME Joel G. Ackerman REGISTRATION NO. (*if appropriate*) 24,307

TELEPHONE 415-576-0200 Docket Number: 018062-006200US

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

SF 1451126 v1

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/15 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number 018062-006200US

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Kevan	Shokat	San Francisco, CA
Chao	Zhang	San Francisco, CA

Number 2 of 2

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SF 1451126 v1

[redacted]

Application Data Sheet

Application Information

Application number::	
Filing Date::	04/11/03
Application Type::	Provisional
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R??::	
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	SELECTIVE SERINE/THREONINE KINASE INHIBITORS
Attorney Docket Number::	018062-006200US
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	25
Small Entity?::	Yes
Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	National Institutes of Health A144009
Secrecy Order in Parent Appl.::	No

Initial 4/11/03

[redacted]

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Jack
Middle Name::	
Family Name::	Taunton
Name Suffix::	
City of Residence::	San Francisco
State or Province of Residence::	CA
Country of Residence::	US
Street of Mailing Address::	2920 22nd Street
City of Mailing Address::	San Francisco
State or Province of mailing address::	CA
Country of mailing address::	
Postal or Zip Code of mailing address::	94110

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Michael
Middle Name::	
Family Name::	Cohen
Name Suffix::	
City of Residence::	San Francisco
State or Province of Residence::	CA
Country of Residence::	US
Street of Mailing Address::	166 Locksley Avenue, Apt. 1
City of Mailing Address::	San Francisco
State or Province of mailing address::	CA

Initial 4/11/03

[redacted]

Country of mailing address::
Postal or Zip Code of mailing address:: 94122

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Kevan
Middle Name::
Family Name:: Shokat
Name Suffix::
City of Residence:: San Francisco
State or Province of Residence:: CA
Country of Residence:: US
Street of Mailing Address:: 783 35th Avenue
City of Mailing Address:: San Francisco
State or Province of mailing address:: CA
Country of mailing address::
Postal or Zip Code of mailing address:: 94121

Applicant Authority Type:: Inventor
Primary Citizenship Country:: Peoples Republic of China
Status:: Full Capacity
Given Name:: Chao
Middle Name::
Family Name:: Zhang
Name Suffix::
City of Residence:: San Francisco
State or Province of Residence:: CA
Country of Residence:: US
Street of Mailing Address:: 1515 27th Avenue
City of Mailing Address:: San Francisco

Initial 4/11/03

[redacted]

State or Province of mailing address:: CA
Country of mailing address::
Postal or Zip Code of mailing address:: 94122

Correspondence Information

Correspondence Customer Number:: 20350

Representative Information

Representative Designation::	Representative Number::	Representative Name::
Primary	24,307	Joel G. Ackerman
Associate	28,219	M. Henry Heines

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Information

Country::	Application number::	Filing Date::

Assignee Information

Assignee Name::
Street of mailing address::
City of mailing address::
State or Province of mailing address::
Country of mailing address::
Postal or Zip Code of mailing address::

60462554.041103

Attorney Docket No.: 018062-006200US

Client Reference No.: SF03-057

PROVISIONAL

PATENT APPLICATION

SELECTIVE SERINE/THREONINE KINASE INHIBITORS

Inventor(s): Jack Taunton, a citizen of The United States, residing at
2920 22nd Street, San Francisco, CA 94110

Michael Cohen, a citizen of The United States, residing at
166 Locksley Avenue, Apt. 1, San Francisco, CA 94122

Kevan Shokat, a citizen of The United States, residing at
783 35th Avenue, San Francisco, CA 94121

Chao Zhang, a citizen of China, residing at
1515 27th Avenue, San Francisco, CA 94122

Assignee: The Regents of the University of California
Office of Technology Management, University of California, San Francisco,
185 Berry Street, Suite 4603
San Francisco, CA, 94107

Entity: small

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 415-576-0200

SELECTIVE SERINE/THREONINE KINASE INHIBITORS

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This work was supported in part by National Institutes of Health grant no. A144009. The government of the United States of America may have certain rights in this invention.

BACKGROUND OF THE INVENTION

[0002] Protein kinases are enzymes that catalyze the transfer of the terminal phosphate group of ATP to serine, threonine, or tyrosine residues on protein substrates. Protein kinases are critical regulators of all cellular processes and play especially important roles in signal transduction pathways that control cell growth and differentiation. Because of their central roles in cell regulation, protein kinases have emerged as attractive drug targets for cancer and inflammatory diseases. There are over 500 protein kinases encoded by the human genome, and each one is a potential drug target.

[0003] One attribute that makes protein kinases attractive drug targets is their ATP-binding site, a deep, hydrophobic cleft at the interface of two conserved subdomains. Many small molecules have been discovered that bind to this site with high affinity. However, because the ATP-binding sites of all protein kinases are highly similar, it has been difficult to design selective inhibitors that specifically target one or a few of the 500 human protein kinases.

[0004] The Rsk serine/threonine protein kinases are thought to have critical functions in the Ras/MAP kinase signaling pathway, a pathway which is deregulated in many human cancers. Of the four Rsk isoforms (Rsk1-4), Rsk1 and Rsk2 are the best characterized. Rsk1 and Rsk2 are directly activated by the MAP kinases, ERK1 and ERK2. Known substrates of Rsk1,2 include transcription factors involved in cell growth and differentiation (e.g. CREB, *c*-fos, estrogen receptor) and apoptosis (NF-κB). Rsk1,2 have thus been implicated in transcriptional control downstream of Ras and ERK1,2.

[0005] Rsk1-4 are unusual protein kinases in that they have two kinase domains, the NTD (NH_2-terminal domain) and the CTD (CO_2H-terminal domain). All Rsk substrates that have been characterized thus far are phosphorylated by the NTD. Downstream signaling by the NTD requires at least three sequential phosphorylation events: (1) phosphorylation of the CTD activation loop (T573) by ERK1,2; (2) intramolecular phosphorylation of a linker region (S380) by the CTD, which creates a docking site for the kinase PDK1; (3) phosphorylation of the NTD activation loop (S221) by PDK1.

BRIEF SUMMARY OF THE INVENTION

[0006] This invention relates generally to the inhibition of protein kinases, and includes inhibitors that specifically target certain protein kinases, as well as the engineering or modification of proteins so as to be susceptible to inhibition by such inhibitors. For example, this invention provides for the modification of proteins for which inhibitors have not yet been identified so that that inhibition of such proteins may be conducted, and the functioning of kinases in signaling networks can be studied and elucidated.

[0007] In one aspect, this invention relates to certain novel compounds that inhibit one or more of the protein kinases referred to herein. These compounds have a heterocyclic core structure comprised of two or more fused rings containing at least one nitrogen ring atom, and an electrophilic substituent that is capable of reacting with a cysteine residue within the ATP binding site of the kinase. Optionally the inhibitors may contain a second group that is capable of reacting with a threonine or smaller residue that is located in the gatekeeper position. This aspect also includes novel pharmaceutical or therapeutic compositions containing effective kinase-inhibitory amounts of such compounds.

[0008] In another aspect this invention relates to a method of inhibiting (preferably inhibiting by formation of a covalent bond that is either reversible or irreversible, and most preferably irreversible) a protein kinase that has one or more cysteine residues within its ATP binding site, comprising contacting the kinase with an inhibitory-effective amount of a compound as described herein.

[0009] In a third aspect this invention involves the engineering or modification of a protein kinase by replacing a valine residue within the ATP binding site of the protein kinase with a cysteine residue. This can render the enyzmatic activity of the modified protein kinase susceptible to inhibition by the compounds disclosed herein.

[0010] In a fourth aspect, this invention involves the engineering or modification of a protein kinase that contains a cysteine in the ATP-binding site corresponding to Cys436 of Rsk2. These kinases include Rsk3, Msk1-2, Plk1-3, MEKK1, and Nek2. The engineering or modification of the protein kinase is achieved by replacing a methionine, leucine, isoleucine, lysine, arginine, tryptophan, glutamine, asparagine, proline, tyrosine, histidine, glutamic acid, aspartic acid, valine, or phenylalanine residue in the gatekeeper position of the ATP binding site with a smaller residue, e.g. a threonine, serine, alanine, or glycine residue. This can render the kinase susceptible to inhibition by some compounds of this invention, and serve to identify such compounds.

[0011] Engineering or modification of a protein kinase can transform a kinase that has no known inhibitors (or that can be only inhibited reversibly) into one that for the first time can be inhibited (or can be inhibited irreversibly), for example by compounds of this invention . The now modified kinase can be used to elucidate kinase functioning in signaling networks, for example by being introduces into genetically transformed animals.

[0012] In other aspects the invention relates to methods for screening candidate compounds for activity as inhibitors of such kinases, to libraries such as combinatorial libraries containing compounds that either have been found to be inhibitors of these kinases or are to be used for screening for such activity, and to products such as arrays, microarrays and the like, that may be used to ascertain protein kinases that bind to and/or are inhibited by, the compounds.

[0013] By "inhibitors" is meant compounds that, *e.g.*, bind to, partially or totally block stimulation, decrease, prevent, or delay activation, or inactivate, desensitize, or down-regulate signal transduction.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 depicts a general scheme for synthesis of pyrrolopyrimidine-based electrophilic inhibitors according to this invention.

[0015] Figure 2 depicts the structure of PP1 bound to Hck (Kuriyan, 1999) showing the proximity of catalytic lysine.

[0016] Figure 3 illustrates that the conformation of the catalytic lysine is different in the active state of Src-kinases.

[0017] Figure 4 depicts an electrophilic fluoromethylketone trap for the catalytic lysine .

[0018] Figure 5 depicts a synthesis route for a pyrrolopirimidine scaffold of the invention.

[0019] Figure 6 depicts palladium cross-coupling reactions used to functionalize the scaffold.

[0020] Figure 7 depicts a scheme for synthesis of halomethylketones of the invention.

[0021] Figure 8 depicts the crystal structure of PP1 bound to Hck, showing the proximity (3.7 Angstroms) of valine-284 (Src numbering) to the pyrazole ring nitrogen of PP1.

[0022] Figure 9 depicts the chloromethylketone pyrrolopyrimidine of the invention bound to the valine(284)- to-cysteine mutant of a Src kinase, showing the proximity of the engineered cysteine to the electrophile.

[0023] Figure 10 depicts and tested for time-dependent inhibition Val 282 of Fyn (a Src-family kinase) that was mutated to Cys by the chloromethyl- and bromomethylketones of the invention.

[0024] Figure 11 depicts a structure-based sequence alignment showing five kinases with cysteines corresponding to Val284 of Src. Only one of these kinases (Rsk2) has a Thr corresponding to Thr341 of Src.

[0025] Figure 12 depicts PP1 bound to Hck with a Met (red mesh) in the gatekeeper position instead of a Thr (yellow).

[0026] Figure 13 shows regulation of Rsk kinases by multiple phosphorylation steps, including autophosphorylation by the C-terminal kinase domain, which is the target of the inhibitors of this invention.

[0027] Figures 14 and 15 contain data from the treatment of cytosolic extracts prepared from Xenopus eggs with 0-5 μM unlabeled chloromethylketone (cmk) for 30 min. at room temperature. Biotinylated cmk was then added at 1 μM for 30 min. Biotinylated proteins were detected on immunoblots with streptavidin-horseradish peroxidase (top panel). Note that there is a high background of proteins that react with the streptavidin-HRP in the untreated control. To test whether the 90kD biotinylated protein was Rsk2, we immunoprecipitated Rsk2 from the cytosol with a monoclonal antibody (Santa Cruz Biotech). Immunoprecipitates were then probed on immunoblots with streptavidin-HRP or with anti-Rsk2 antibody (lower panels).

[0028] Figure 16 shows a synthesis scheme for bodipy-cmk. The same procedure was used to prepare biotinylated-cmk using biotin-NHS ester.

[0029] Figure 17 shows data from a repeat of the experiment described in Figures 14 and 15, using bodipy-cmk instead of biotin-cmk. In addition, the unlabeled enoate was tested for its ability to bind to p90. Here, labeled proteins were detected by a scanning confocal laser. p90 is the only band that disappears in the presence of unlabeled cmk and enoate.

[0030] Figure 18: the lower panel shows the same gel as in Fig. 17, but now all of the cytosolic proteins are revealed by Coomassie staining. The bodipy-cmk is remarkably specific for p90Rsk.

[0031] Figure 19 shows inhibition of Rsk autophosphorylation in Swiss 3T3 fibroblasts. The enoate blocks Rsk autophosphorylation of Ser381, which is catalyzed by its cysteine-containing C-terminal kinase domain. The enoate does not block Erk phosphorylation. Swiss 3T3 cells were serum starved for 24 hours and treated with 5 μM enoate or 0.1% DMSO (control) for 1 hour. The cells were then stimulated with growth factors for 10 min. and lysed with 1x SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Phosphorylation-specific antibodies against Rsk and Erk were purchased from Cell Signaling. Antibodies against nonphosphorylated Rsk and Erk were purchased from Santa Cruz Biotech and Cell Signaling, respectively.

[0032] Figure 20 depicts a comparison of PP1 bound to Hck and the chloromethyl ketone (cmk) of this invention bound to V282C. Both compounds have a bulky group at the 3-position but only the cmk has an electrophile.

[0033] Figure 21 depicts a synthesis route for halomethyl ketones.

[0034] Figure 22 is a graphical depiction of data from an experiment in which V282C Fyn was incubated with 10 nM bmk **3** or cmk **4** for varying times. *In vitro* kinase assays were then performed. The graph demonstrates that bmk **3** or cmk **4** exhibit time-dependent inactivation of kinase activity.

[0035] Figure 23 shows: (A) Chemical structure of biotin-cmk. (B) NIH3T3 fibroblasts expressing either v-Src-cs1 or wt v-Src were pretreated with increasing concentrations of cmk **4** for 2hrs. Whole cell lysates were prepared and treated with biotin-cmk(2 μM) for 1 hr prior to immunoprecipitation of v-Src. Immunoprecipitated proteins were resolved by SDS-PAGE

and detected by western blot analysis with anti-v-Src mAb (327) and/or streptavidin-horseradish peroxidase conjugate.

[0036] Figure 24 depicts results from an experiment in which NIH3T3 fibroblasts expressing either v-Src-es1 or wt v-Src were pretreated with increasing concentrations of cmk 4 for 2hrs. Cells were subsequently washed free of cmk 4 with warmed compound-free medium and incubated for 1 hr. Whole cell lysates were prepared and tyrosine phosphorylated proteins were resolved by SDS-PAGE and analyzed by western blot with anti-phosphotyrosine mAb (4G10).

[0037] Figure 25 depicts results from an experiment in which: (A) Cells expressing either wt v-Src or v-Src-es1 were treated with 1μM cmk 4 for 16 h at 37 0C. Cells were fixed, stained with phalloidin-FITC and visualized by epifluorescence microscopy. (B) 100-200 cells from each treatment condition in (A) for both wt v-Src and v-Src-cs1 were categorized as having: flat morphology, round morphology, or inconclusive morphology.

DETAILED DESCRIPTION OF THE INVENTION

[0038] This invention describes cell-permeable inhibitors that exploit a feature unique to the ATP-binding sites of three closely related kinases (Rsk1, Rsk 2, and Rsk 4; 80-90% sequence identity) and that also is present in certain other kinases. More specifically, Rsk1,2,4 are unique among kinases in having a cysteine and a threonine in their ATP-binding sites. These constitute specific recognition elements for the inhibitors described in this invention. As described below, the compounds of this invention inhibit activity of these three kinases by binding to the cysteine and the threonine. In other instances, as described below, the compounds may inhibit kinase activity by binding to a cysteine, while binding of the compound to a threonine or other residue might or might not take place.

[0039] The invention also describes a method for identifying inhibitors for eight other human protein kinases (Rsk3, Msk1,2, Plk1-3, MEKK1, and Nek2), based on the presence of a key cysteine residue in their ATP-binding sites analogous to the cysteine found in Rsk1,2,4. At least two of these kinases, Plk1 and Nek2, are candidate anti-cancer targets based on their essential mitotic functions in model organisms. To the best of our knowledge, there are no published or patented inhibitors that specifically target any of these protein kinases.

[0040] Thus, compounds of this invention have a heterocyclic core structure comprised of two or more fused rings containing at least one nitrogen ring atom, and an electrophilic substituent that is capable of reacting with a cysteine residue within the ATP binding site of the kinase. Optionally they may contain a second group that is capable of reacting with a threonine or smaller residue that is located in the gatekeeper position.

[0041] The Rsk serine/threonine protein kinases are thought to have critical functions in the Ras/MAP kinase signaling pathway, a pathway which is deregulated in many human cancers. Of the four Rsk isoforms (Rsk1-4), Rsk1 and Rsk2 are the best characterized. Rsk1,2 are directly activated by the MAP kinases ERK1 and ERK2. Known substrates of Rsk1,2 include transcription factors involved in cell growth and differentiation (e.g. CREB, *c*-fos, estrogen receptor) and apoptosis (NF-κB). Rsk1,2 have thus been implicated in transcriptional control downstream of Ras and ERK1,2.

[0042] Rsk1-4 are unusual protein kinases in that they have two kinase domains, the NTD (NH_2-terminal domain) and the CTD (CO_2H-terminal domain). All Rsk substrates characterized thus far are phosphorylated by the NTD. Downstream signaling by the NTD requires at least three sequential phosphorylation events: (1) phosphorylation of the CTD activation loop (T573) by ERK1,2; (2) intramolecular phosphorylation of a linker region (S380) by the CTD, which creates a docking site for the kinase, PDK1; (3) phosphorylation of the NTD activation loop (S221) by PDK1. This invention describes inhibitors that specifically target the Rsk CTD, which therefore indirectly inhibit downstream signaling by the NTD.

[0043] In one aspect, this invention relates to certain novel compounds that inhibit one or more of the protein kinases referred to herein, the compounds having a heterocyclic core structure comprised of two or more fused rings containing at least one nitrogen ring atom, and an electrophilic substituent that is capable of reacting with a cysteine in the ATP binding site of Rsk1-4, Msk1-2, Plk1-3, MEKK1, and Nek2, as shown in Sequence I below.

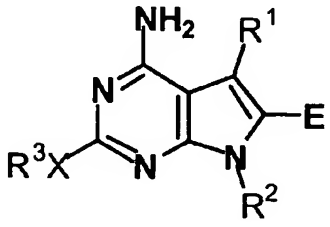
[0044] In another aspect this invention relates to a method of inhibiting (preferably inhibiting by formation of a covalent bond that is either reversible or irreversible, and most preferably irreversible) a protein kinase that has one or more cysteine residues within its ATP binding site, and more particularly a cysteine corresponding to the cysteine found in

Rsk2, Nek2, MEKK1, Msk1, and Plk1 (see Sequence I below) comprising contacting the kinase with an inhibitory-effective amount of a compound as described herein.

[0045] In other aspects the invention relates to methods for screening candidate compounds for activity as inhibitors of the kinases, to libraries such as combinatorial libraries containing compounds that either have been found to be inhibitors of these kinases or are to be used for screening for such activity, and to products such as arrays, microarrays and the like, that may be used to ascertain proteins that bind to and/or are inhibited by, the compounds.

[0046] The compounds that have been found to inhibit the eight kinases, and that thus form one aspect of this invention, are compounds that have a heterocyclic core, preferably a core composed of two or more fused rings (most preferably from 2-5 fused rings) containing at least one nitrogen ring atom, and that have an electrophilic ring substituent. In addition to the one or more nitrogen atoms the compounds may also have one or more other heterocyclic ring atoms such as oxygen or sulfur.

[0047] More particularly, the compounds that are effective as inhibitors of the kinases include those having the formula (I) or formulas (II-V) shown below. Compounds of formula (I) are considered to be optimal for inhibiting Rsk-1,2,4 while compounds of formulas (II)-(V) are considered optimal for inhibiting Rsk3, Msk1,2, Plk1-3, MEKK1, and Nek2, which contain a cysteine homologous to Cys 436 of Rsk2. In Formula (I) below, R^1 generally is an aromatic or heteroaromatic group such as phenyl optionally substituted with lower alkyl, halogen, substituted alkyl, nitro, alkoxy, phenoxy, etc. groups; E is an electrophilic group; R^2 is generally an aliphatic, aromatic, or heteroaromatic group optionally substituted with polar groups such as alcohols, amines, or nitrogen-containing heterocycles; R^3 X is generally hydrogen but can be an alkyl- or aryl-substituted ether, thioether, or amine.



(I)

[0048] The pyrrolopyrimidines can also be viewed as in Formula (IA), which illustrates functions for the purpose of this invention, of certain aspects of the structures of pyrrolopyrimidines.

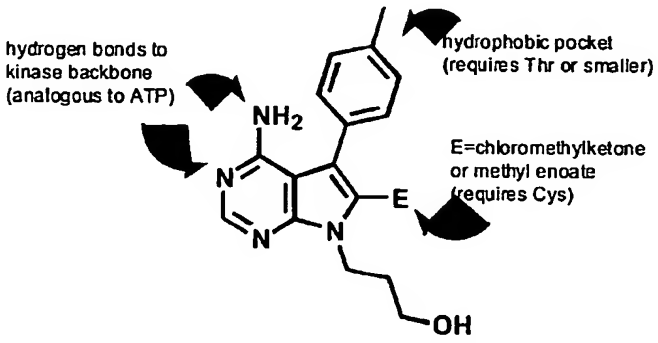
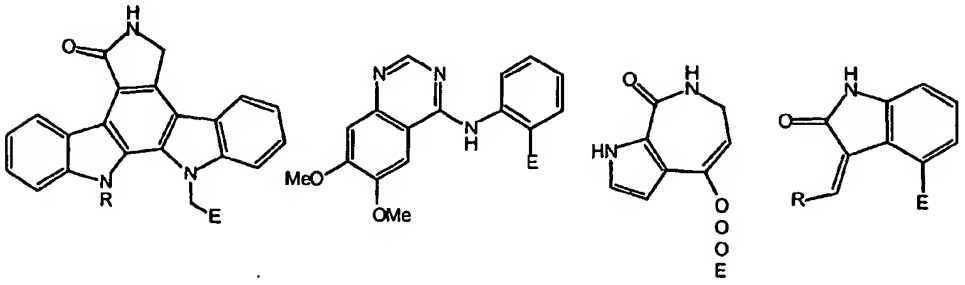


Fig. 1. Rsk inhibitors based on a pyrrolopyrimidine scaffold.

(IA)

[0049] Structures of other compounds that my be used for inhibition of these kinases and are optimal for Rsk3, Msk1,2, Plk1-3, MEKK1, and Nek2, include, for instance,



(II) **(III)** **(IV)** **(V)**

[0050] In formulas (I) - (V) E represents an electrophilic moiety as described herein. Preferably, for optimum inhibitory effect this group is located on the core heterocyclic ring in the positions shown. However, in compounds of this invention electrophilic group E may be located at other positions on the respective rings, as shown for example in Figure 1.

[0051] The electrophilic group E is most preferably one that comprises a carbonyl, an epoxide, or an olefin conjugated to an electron withdrawing group such as a carbonyl, nitro, cyano, sulfoxide, or sulfonyl. For carbonyl groups, ketonic groups RCOR' are preferred (R and R' may be the same or different). Groups that form "haloketones", such as –C(O)Cl, -C(O)F, $-CH_2C(O)Cl$,$-CH_2C(O)F$ (acyl halides), $-C(O)CH_2Cl$, $-C(O)CH_2Br$ (halomethyl ketones), and that form olefinically unsaturated ketones, such as $-C(O)CH=CH_2$, are preferred.

[0052] Olefin carboxylates have the general formula $–CH=CHC(O)OR_1$ where R_1 is an optionally substituted aliphatic, aromatic, or heterocyclic moiety. Also preferred are olefin carboxamides of general formula $-CH=C(O)NR_1R_2$ where R_1 and R_2 are optionally substituted aliphatic, aromatic, or heterocyclic moieties.

Inhibitor design

[0053] Rsk inhibitors were designed to occupy the ATP-binding pocket based on several key interactions with a pyrrolopyrimidine scaffold (Fig. 1; Formula IA) [again, do the compounds have to have several interactions in order to be considered within the invention, or is one interaction sufficient?]. Using a structure-based sequence alignment (Sequence I; Fig. 11), we predicted [found?] that Cys 436 in human Rsk2 would be suitably oriented to react with an electrophilic moiety such as a chloromethylketone or a methyl enoate attached to C2 of a pyrrolopyrimidine scaffold (Formula IA). This is the most crucial specificity element of the inhibitor, as only 11 of the 518 human kinases (Rsk1-4, Msk1,2, Plk1-3, MEKK1, and Nek2) have a cysteine in this position.

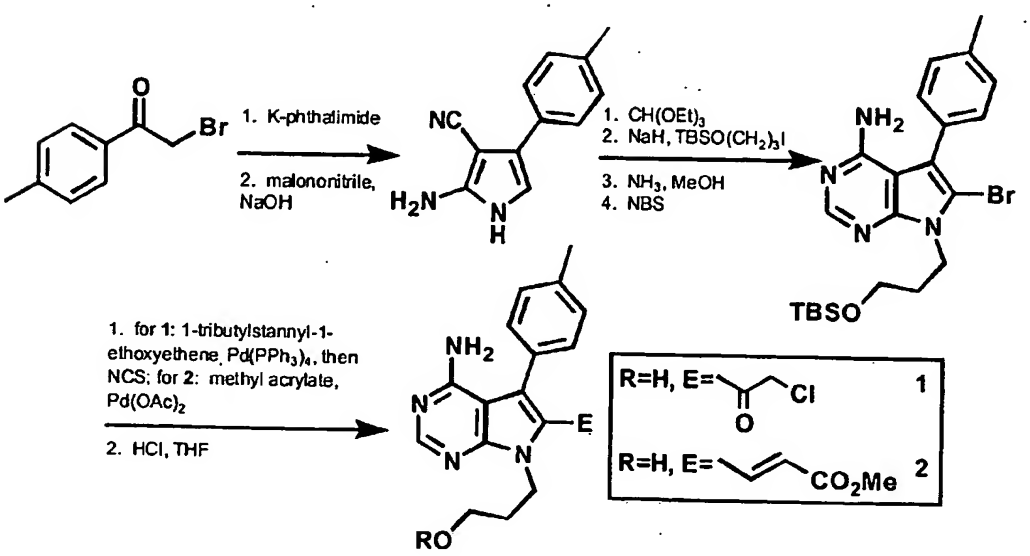
```
src     -------LRLEVK-LGQGCFGEVWMGTWNG--TTRVAIKTLKPGTMS---
rsk2    -IQFTDGYEVKED-IGVGSYSVCKRCIHKATN-MEFAVKIIDKS------
nek2    MPSRVEDYEVLHS-IGTGSYGRCQKIRRKSDG-KILVWKELDYGSMT---
mekk1   --REDTEWLKGQQ-IGLGAFSSCYQAQDVGTG-TLMAVKQVTYVRNTSSE
msk1    ----HYDLDLKDKPLGEGSFSICRKCVHKKSN-QALQVKIISKR------
plk1    -----RRRYVRGRFLGKGGFAKCFEISDADTK-EVFAGKIVPKSLLLK--

src     ----PEAFLQEAQVMK--KLRHEKLVQLYAVVSEEP---IYIVTEYM
rsk2    ----KRDPTEEIEILLR-YGQHPNIITLKDVYDDGKY--VYVVTELM
nek2    -EVEKQMLVSEVNLLR--ELKHPNIVRYYDRIIDRTNTTLYIVMEYC
mekk1   QEEVVEALREEIRMMS--HLNHPNIIRMLGATCEKSN--YNLFIEWM
msk1    ---MEANTQKEITALK-LCEGHPNIVKLHEVFHDQLH--TFLVMELL
plk1    -PHQREKMSMEISIHR--SLAHQHVVGFHGFFEDNDF--VFVVLELC
```

Sequence I. Structure-based sequence alignment of kinase domains with a cysteine predicted to interact with C2-substituted electrophilic pyrrolopyrimidines. The tyrosine kinase Src, which binds a structurally related inhibitor PP1, is shown for reference. Cysteine, valine, methionine, leucine and threonine referred to in the specification are highlighted.

[0054] We also determined that Thr 493 of Rsk2 could accommodate a large aromatic substituent at C3 of the pyrrolopyrimidine scaffold and could form a hydrogen bond with the exocyclic amine, by analogy to known tyrosine kinase inhibitors (e.g. PP1). With the exception of Rsk1,2,4, all of the aforementioned kinases have a large amino acid at this position (methionine, leucine, or isoleucine). Thus, Rsk1,2,4 are the only protein kinases that we believe are irreversibly inhibited by the compound shown in Formula IA.

Inhibitor synthesis

[0055] Electrophilic Rsk inhibitors **1** and **2** were synthesized in eight and seven steps, respectively, starting from *p*-methyl-α-bromoacetophenone, as shown and described below (Scheme A; Figures 5). Inhibitors **3-5** were synthesized as shown in Figure 21. Biotin-**1** was prepared by reacting **1** with biotin-aminocaproic acid in the presence of MSNT. All intermediates were characterized by mass spectrometry and ^{1}H NMR.

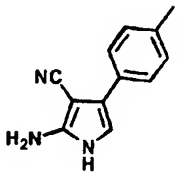


Scheme A. Synthesis of Rsk inhibitors **1** and **2**.

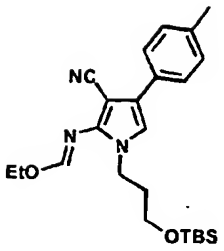
Experimental section

Materials. All solvents were of ACS chemical grade (Fisher) and were used without further purification unless otherwise indicated. Methylene chloride was dried by distillation from calcium hydride. Tetrahydrofuran was distilled from sodium/benzophenone ketyl. All starting materials and synthetic reagents were purchased from commercial sources unless otherwise noted.

General Experimental. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer at 400 and 100 MHz, respectively. Low-resolution electrospray ionization mass spectra were recorded on a Waters ZQ 4000. High-resolution electron impact mass spectra were recorded on a MicoMass VG70E spectrometer by Yuequan Sun at the University of California-San Francisco center for Mass Spectrometry. Analytical and preparative thin layer chromatography was preformed with EM Science silica gel 60 F_{254} glass plates. Flash chromatography was conducted with Merck silica gel 60 (230-400 mesh).

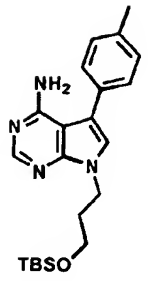


Step 1. To a solution of malononitrile (3.24 g, 48.9 mmol) in 6:1:1 MeOH/48% aq. NaOH/H_2O (56 mL) was added 2-phthalamido-4'-methylacetophenone[1] (10.5 g, 37.6 mmol). The reaction mixture was stirred at room temperature for 1 h upon which the product precipitated out of solution. The solid was collected by filtration, washed with H_2O, CH_2Cl_2, and hexanes to give 6.7 g (91% yield) of the pyrrole as a brown solid: R_f 0.70 (10% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, CD_3OD) δ7.409 (d, J = 7.6 Hz, 2H), 7.08 (d, J = 7.6 Hz, 2H), 6.36 (s, 1H), 4.83 (br s 2H), 2.27 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) (partial) δ136.9, 130.1, 126.6, 120.1, 109.0, 21.1.



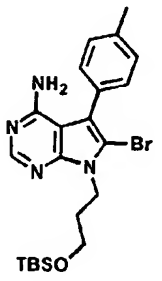
Step 2. A solution of the pyrrole (5 g, 25.3 mmol) in triethyl orthoformate (30 mL) was treated with acetic anhydride (0.5 mL) and refluxed for 1 h. After cooling to room temperature, the solvent was removed in vacuo. The crude pyrrole imino ether was azeotropically dried with toluene (2 x 10 mL) and carried on directly to the next step.

To a solution of NaH (60% in oil, 0.9 g, 23.4 mmol) in 20 mL of DMF at room temperature was added a solution of pyrrole imino ether in 10 mL of DMF. After stirring for 30 min, 3-(*t*-butyldimethylsilyloxy)propyl iodide[2] (7.0 g, 23.4 mmol) was added over 10 min. After stirring for an additional 3 h, the solvent was removed in vacuo and the residue was purified by flash chromatography (10-50% ethyl acetate/hexanes) to afford 5 g (70% yield) of the N-alkyl pyrrole imino ether as a brown oil: R_f 0.8 (4:1 hexanes/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ8.43 (s, 1H), 7.47 (d, J = 8.0 Hz, 2H), 7.16 (d, J = 8.0 Hz, 2H), 6.62 (s, 1H), 4.32 (q, J = 8.0 Hz, 2H), 3.96 (t, J = 7.2 Hz, 2H), 3.59 (t, J = 5.6 Hz, 2H), 2.33 (s, 3H), 1.91-1.82 (m, 2H), 1.37 (t, 3H), 0.88 (s, 9H), 0.03 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ158.2, 144.1, 136.8, 130.5, 129.7, 126.2, 125.0, 118.5, 115.6, 63.4, 59.7, 43.1, 33.4, 26.1, 21.4, 18.5, 14.2.



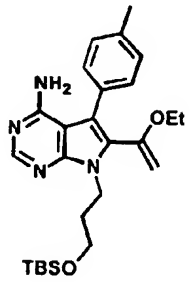
A

Pyrrolo[2,3-*d*]pyrimidine A. The imino ether from step 2 (18.0 g, 42.3 mmol) was dissolved in MeOH (100 mL) and transferred to 350 mL sealed-tube reaction vessel. Argon gas was then bubbled through the solution for 15 min. Reaction vessel was submerged in a dry ice/MeOH bath and NH_3 gas was bubbled through the solution for 15 min to saturate. The reaction vessel was quickly sealed with a Teflon screw cap (fitted with a rubber O-ring) and allowed to warm to room temperature. The reaction was stirred for 4 days at 50 ^{0}C and then submerged in an ice bath and NH_3 gas was slowly released. The solvent was removed in vacuo and the crude product was purified by flash chromatography (3:2 ethyl acetate/hexanes with 1% Et_3N) to give 10.1 g (61% yield) of **A** as a light-brown solid: R_f 0.41 (1:1 hexanes/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) 8.32 (s, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 6.92 (s, 1H), 5.1 (br s, 2H), 4.34 (t, J = 6.8 Hz, 2H), 3.65 (t, J = 6.0 Hz, 2H), 2.41 (s, 3H), 2.10-2.05 (m, 2H), 0.91 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ156.9, 151.9, 150.6, 136.8, 132.0, 129.7, 128.7, 123.2, 115.8, 101.3, 59.8, 41.5, 33.0, 25.9, 21.1, 18.2; ESI-MS 419 $[M+Na]^+$, 397 $[M+H]^+$.



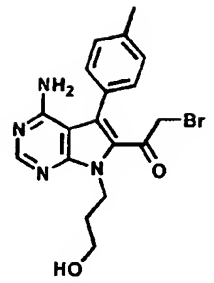
B

Bromide B. To a solution of pyrrolo[2,3-*d*]pyrimidine **A** (1.5 g, 3.78 mmol) in DMF (20 mL) was added NBS (0.74 g, 4.16 mmol) and the mixture stirred for 24 h in the absence of light. The reaction was diluted with ether (100 mL) and washed with water (3 x 200 mL). The combined aqueous fractions were extracted with ether (3 x 50 mL). The combined organic fractions were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give 1.77 g (99% yield) of bromide **B** as a brown solid: R_f 0.59 (100% ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) □8.21 (s, 1H), 7.35 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 5.0 (br s, 2H), 4.41 (t, J = 6.8 Hz, 2H), 3.73 (t, J = 6.0 Hz, 2H), 2.43 (s, 3H), 2.1 (m, 2H), 0.91 (s, 9H), 0.06 (s, 6H); ESI-MS 500 $[M+2+Na]^+$, 497 $[M+Na]^+$, 477 $[M+2]^+$, 475 $[M]^+$.



C

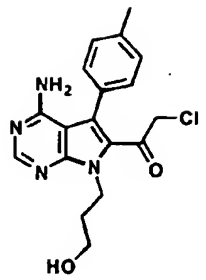
Enol ether C. To a solution of bromide **B** (389 mg, 0.82 mmol) in toluene (10 mL) was added α-(ethoxyvinyl)tin (0.390 mL, 1.15 mmol). Argon gas was bubbled through the solution for 10 min. Tetrakis(triphenylphosphine)palladium (95 mg, 0.082 mmol) was quickly added and mixture was refluxed for 16 h. The solvent was removed in vacuo and the crude product was purified by flash chromatography (50-100% hexanes/ethyl acetate) to give enol ether **C** (304 mg, 80% yield) as solid: R_f 0.25 (1:1 hexanes/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ8.31 (s, 1H), 7.31 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 8.0 Hz, 2H), 4.99 (br s, 2H), 4.39 (s, 1H), 4.37 (t, 2H), 4.17 (s, 1H), 3.84 (q, J = 6.8 Hz, 2H), 3.72 (t, 2H), 2.40 (s, 3H), 2.1 (m, 2H), 1.34 (t, 3H), 0.89 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ157.3, 152.4, 152.0, 150.2, 137.1, 131.9, 130.9, 130.2, 129.4, 114.9, 101.7, 91.6, 63.6, 61.2, 41.2, 33.5, 26.1, 21. 4, 18.5, 14.6; ESI-MS 489 $[M+Na]^+$, 467 $[M+H]^+$.



3

bmk 3. To a solution of enol ether **C** (81 mg, 0.17 mmol) in DMF (2 mL) and H_2O (0.006 mL) at –20 ^{0}C was added $NaHCO_3$ (22 mg, 0.26 mmol). NBS (31 mg, 0.17 mmol) was quickly added and mixture was stirred at room temperature in the dark. After 15 min, the reaction was diluted with ethyl acetate (20 mL) and washed with saturated Na_2SO_3 (1 x 10 mL) and H_2O (1 x 10 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Rapid purification by flash chromatography (1:1 hexanes/ethyl acetate) gave 52 mg (60% yield) of bmk silyl ether, which was used without further purification.

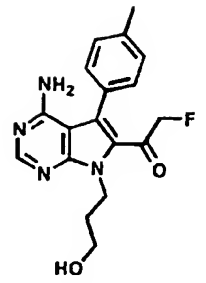
To a solution of the bmk silyl ether (18 mg, 0.035 mmol) in THF (1 mL) at 0 ^{0}C was added 1 N HBr (0.33 mL). After stirring for 30 min at 0 ^{0}C and 2 h at room temperature, the reaction was diluted with ethyl acetate (5 mL) and washed with saturated $NaHCO_3$ (1 x 5 mL) and H_2O (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by preparative HPLC (30-100% MeOH gradient over 15 min; 10 mL/min flow rate; retention time for **3** was 11.9 min), afforded the desired bmk **3** as an off-white solid: ^{1}H NMR (400 MHz, $CDCl_3$) □ 8.36 (s, 1H), 7.38 (br s, 4H), 5.0 (br s, 2H), 4.66 (t, J = 5.6 Hz, 2H), 3.74 (s, 2H), 3.46 (m, 2H), 2.49 (s, 3H), 2.12-2.07 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ185.4, 159.0, 155.6, 151.9, 139.9, 130.4, 130.3, 129.5, 128.1, 124.7, 102.1, 57.7, 40.5, 34.3, 32.9, 21.4; ESI-MS 405 $[M+2]^+$, 403 $[M]^+$; HRMS (EI) Calcd for $C_{18}H_{19}BrN_4O_2$ 402.0691, found 402.0699.



4

cmk 4. To a solution of enol ether **2** (262 mg, 0.56 mmol) in MeCN (6 mL) and H_2O (0.05 mL) was added $NaHCO_3$ (94 mg, 1.12 mmol). NCS (89 mg, 0.67 mmol) was quickly added and mixture was stirred at room temperature in the dark. After 1 h, the reaction was diluted with ethyl acetate (20 mL) and washed with saturated Na_2SO_3 (1 x 10 mL) and brine (1 x 10 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Rapid purification by flash chromatography (1:1 hexanes/ethyl acetate) gave 101 mg (40% yield) of cmk silyl ether, which was used immediately in the next reaction.

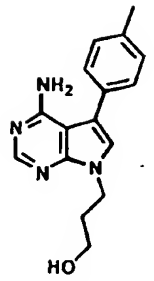
To a solution of the cmk silyl ether (32 mg, 0.046 mmol) in THF (1 mL) at 0 ^{0}C was added 1 N HCl (0.33 mL). After stirring for 30 min at 0 ^{0}C and 2 h at room temperature, the reaction was diluted with ethyl acetate (5 mL) and washed with saturated $NaHCO_3$ (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by preparative HPLC (30-100% MeOH gradient over 15 min; 10 mL/min flow rate; retention time for **4** was 11.2 min), afforded the desired cmk **4** as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ8.36 (s, 1H), 7.37 (d, 2H), 7.36 (d, 2H), 5.0 (br s, 2H), 4.67 (t, J = 6.0 Hz, 2H), 3.93 (s, 2H), 3.40 (m, 2H), 2.49 (s, 3H), 2.10 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) □185.1, 159.1, 155.8, 151.8, 140.0, 130.5, 130.3, 129.5, 128.1, 124.8, 102.1, 57.7, 48.2, 40.6, 32.9, 21.4; ESI-MS 361 $[M+2]^+$, 359 $[M]^+$; HRMS (EI) Calcd for $C_{18}H_{19}ClN_4O_2$ 358.1196, found 358.1196.



5

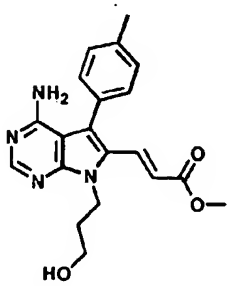
fmk 5. To a solution of bmk silyl ether (22 mg, 0.042 mmol) in MeCN (1 mL) and DMF (0.1 mL) was added KF (19 mg, 0.327 mmol). [bmim][BF_4] (0.15 mL) was added followed by H_2O (0.034 mL) and the reaction was brought to 60 ^{0}C. After 3 h, the reaction was diluted with ethyl acetate (5 mL) and washed with H_2O (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Rapid purification by flash chromatography (1:1 hexanes/ethyl acetate) gave 7.2 mg (40% yield) of fmk silyl ether, which was used immediately in the next step.

To a solution of the fmk silyl ether (7.2 mg, 0.016 mmol) in THF (1 mL) at 0 ^{0}C was added 1 N HCl (0.35 mL). After stirring for 3 h at 0 ^{0}C, the reaction was diluted with ethyl acetate (5 mL) and washed with saturated $NaHCO_3$ (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by preparative HPLC (30-100% MeOH gradient over 15 min; 10 mL/min flow rate; retention time for **5** was 10.3 min), afforded the desired fmk **5** as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ8.37 (s, 1H), 7.38 (d, 2H), 7.34 (d, 2H), 4.95 (br s, 2H), 4.72 (t, J = 5.6 Hz, 2H), 4.68-4.56 (d, J_{HF} = 47 Hz, 2H), 3.46 (m, 2H), 2.49 (s, 3H), 2.13-2.08 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) (partial) □155.8, 140.0, 130.5, 129.3, 57.7, 40.7, 32.9, 21.4; HRMS (EI) Calcd for $C_{18}H_{19}FN_4O_2$ 342.1492, found 342.1502.



6

Pyrrolo[2,3-*d*]pyrimidine 6. To a solution of 1 (49 mg, 0.123 mmol) in THF (3 mL) at 0 ^{0}C was added 1 N HCl (0.5 mL). After stirring for 1 h 30 min at 0 ^{0}C and 30 min at room temperature, the reaction mixture was diluted with ethyl acetate (10 mL) and washed with saturated $NaHCO_3$ (1 x 10 mL) and brine (1 x 10 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to provide 35 mg (100% yield) of **6** as a clear film; Compound **6** was found to be 99% pure by HPLC (30-100% MeOH gradient over 15 min; 0.75 mL/min flow rate; retention time for **6** was 9.3 min); ^{1}H NMR (400 MHz, $CDCl_3$) δ8.27 (s, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.3 Hz, 2H), 6.91 (s, 1H), 5.4 (br s, 2H), 4.35 (t, J = 6.0 Hz, 2H), 3.46 (t, J = 5.6 Hz, 2H), 2.41 (s, 2H), 1.96 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ157.2, 151.8, 150.6, 137.0, 131.6, 129.7, 128.6, 122.7, 116.6, 100.7, 57.5, 40.6, 33.3, 21.1; HRMS (EI) Calcd for $C_{16}H_{18}N_4O_2$ 282.1480, found 282.1477.

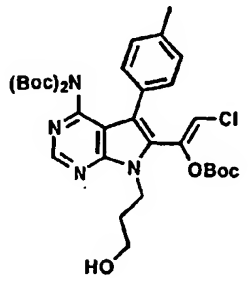


7

Methyl enoate 7. To bromide **1** (137 mg, 0.29 mmol) in DMF (2 mL) and Et_3N (0.20 mL, 1.44 mmol) was added palladium acetate (22.5 mg, 0.1 mmol). The mixture was degassed by two cycles of freeze-pump-thaw and tri-*o*-tolylphosphine (53 mg, 0.17 mmol) was added,

followed by methyl acrylate (0.206 mL, 2.90 mmol). The mixture was heated to 100 ^{0}C in a sealed-tube reaction vessel. After 4 h, the mixture was concentrated in vacuo and the crude product was rapidly purified by flash chromatography (1:1 hexanes/ethyl acetate) to give 70 mg (51% yield) of crude methyl enoate silyl ether, which was used without further purification.

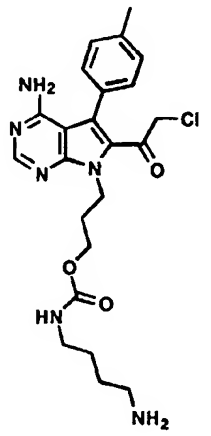
To a solution of the silyl ether (22 mg, 0.046 mmol) in THF (2 mL) at 0 ^{0}C was added 1 N HCl (0.3 mL). After stirring for 1 h 30 min at 0 ^{0}C and 1 h at room temperature, the reaction was diluted with ethyl acetate (5 mL) and washed with saturated $NaHCO_3$ (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by preparative TLC (2 x 0.5 mm plates; 10% MeOH/CH_2Cl_2) afforded 7.3 mg (46% yield) of methyl enoate **7** as a slightly yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ8.29 (s, 1H), 7.61 (d, J = 16.4 Hz, 1H), 7.31 (d, 2H), 7.30 (d, 2H), 6.05 (d, J = 16.4 Hz, 1H), 5.2 (br s, 2H), 4.53 (t, J = 5.6 Hz, 2H), 3.73 (s, 3H), 3.46 (m, 2H), 2.45 (s, 3H), 2.01 (m, 2H).



8

Enol carbonate 8. To a solution of cmk silyl ether (50 mg, 0.11 mmol) in THF (0.60 mL) was added $(Boc)_2O$ (210 mg, 0.42 mmol), followed by DMAP (6.5 mg, 0.053 mmol). After stirring for 2h 40 min at room temperature, the mixture was diluted with ethyl acetate (5 mL) and washed with 10 % citrate buffer pH 4.0 (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to provide 62 mg of the crude enol carbonate silyl ether. This compound was dissolved in THF (1 mL) and treated at 0 ^{0}C with 1 N HCl (0.33 mL). After 3 h, the reaction was diluted with ethyl acetate (5 mL) and washed with saturated $NaHCO_3$ (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, concentrated, and purified by flash

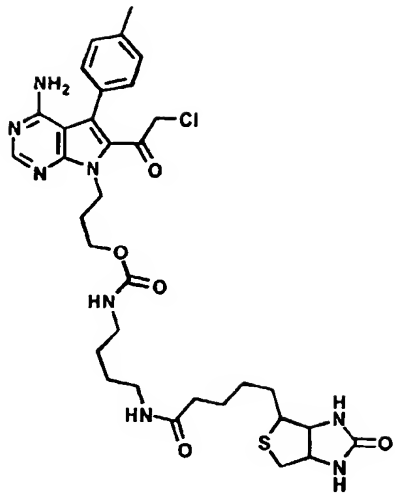
chromatography (50-100 % hexanes/ethyl acetate) to provide the enol carbonate **8** (49 mg, 84% over two steps): R_f 0.40 (1:1 hexanes/ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ8.83 (s, 1H), 7.35 (d, J = 8 Hz, 2H), 7.16 (d, J = 8 Hz, 2H), 5.88 (s, 1H), 4.50 (t, 2H), 3.52 (t, 3H), 2.40 (s, 3H), 2.0 (m, 2H), 1.49 (s, 9H), 1.28 (s, 18H).



9

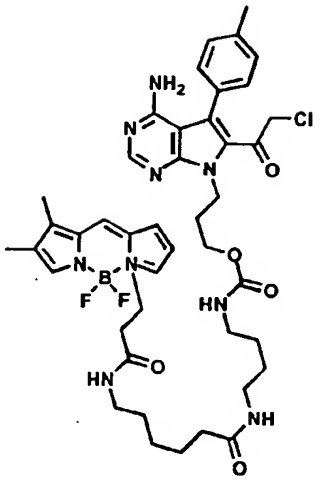
Carbamate 9. Enol carbonate **8** (51 mg, 0.077 mmol) was azeotropically dried with toluene under high vacuum and dissolved in CH_2Cl_2 (0.5 mL). DIPEA (0.015 mL, 0.085 mmol) was added, followed by CDI (14 mg, 0.085 mmol). After stirring for 1 h at room temperature, *N*-Boc-1,4-diaminobutane (16 mg, 0.085 mmol) was added. After 5 h 40 min, and additional 1.1 equivalents of N-Boc-1,4-diaminobutane (16 mg, 0.085 mmol) was added. After 8h, the reaction was diluted with ethyl acetate (5 mL) and washed with 10% sodium citrate buffer (1 x 5 mL) and brine (1 x 5 mL). The organic fraction was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Rapid purification by flash chromatography (1:1-2:1 ethyl acetate/hexanes) gave 43 mg of the boc-protected carbamate, which was used immediately in the next step.

To a solution of the boc-protected carbamate (43 mg, 0.056 mmol) in CH_2Cl_2 (0.8 mL) at 0 ^{0}C was added TFA (0.7 mL). After warming to room temperature and stirring for 3 h, solvents were removed in vacuo to yield 26 mg (73% over three steps) of carbamate **9** as a white solid; ESI-MS 497 $[M+2+Na]^+$, 495 $[M+Na]^+$, 475 $[M+2]^+$, 473 $[M]^+$.



10

Biotin cmk 10. To a solution of carbamatc **9** (9 mg, 0.019 mmol) in DMF (0.1 mL) at room temperature was added Biotin-NHS (13 mg, 0.038 mmol), followed by DIPEA (0.007 mL, 0.038 mmol). After 24 h, the crude mixturc was purificd directly by preparative HPLC (30-100% MeOH gradient over 15 min; 10 mL/min flow rate; retention timc for **10** was 12.2 min) to afford 9 mg (70% yield) of the desired biotin cmk **10** as a white solid; ESI-MS 723 $[M+2+Na]^+$, 721 $[M+Na]^+$.



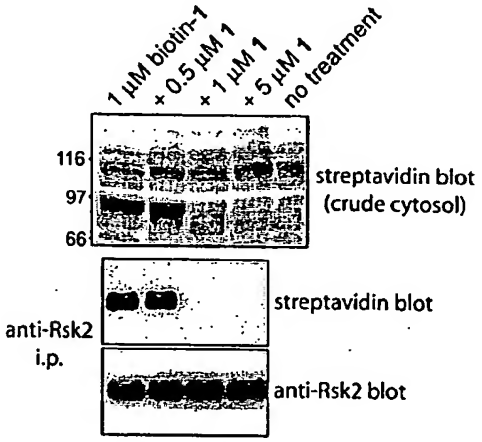
11

Bodipy cmk 11. To a solution of carbamate **9** (4 mg, 0.008 mmol) in DMF (0.1 mL) at room temperature was added Bodipy-NHS (5 mg, 0.009 mmol), followed by DIPEA (0.003 mL, 0.016 mmol). After 24 h in the dark, the solvents were removed and the mixture was purificd by flash chromatography (10:1 CH_2Cl_2/MeOH) to give bodipy cmk **11** as a red solid in quantitative yield; ESI-MS 882 $[M+Na]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ11.2 (br s, 2H), 8.23 (s, 1H), 8.00 (s, 1H), 7.37 (d, J = 8 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 7.1 (s, 1H), 6.86 (d, J = 3.6 Hz, 1H), 6.23 (d, J = 3.6 Hz, 1H), 6.10 (s, 1H), 5.80 (m, 2H), 4.70 (t, 2H), 4.04 (t, 2H), 3.92 (s, 2H), 3.68 (m, 2H), 3.25-3.06 (m, 6H), 2.68 (s), 2.60 (t, J = 7.6 Hz, 2H), 2.53 (s, 3H), 2.46 (s, 3H), 2.23 (s, 3H), 2.1 (m, 2H), 1.46-1.37 (m, 10H).

Inhibitor validation

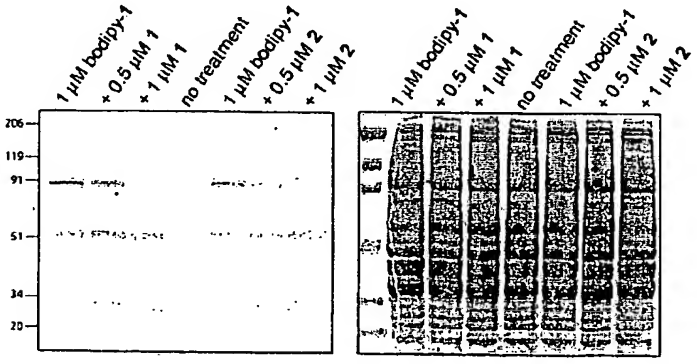
[0056] [We should include details of the procedure. Perhaps this paragraph could be rewritten to include them] To test whether compounds **1** and **2** bind selectively and irreversibly to Rsk2, we labeled compound **1** with biotin (Fig. 3, R=biotin). Xenopus egg cytosol was treated with 1 μM biotin-**1**. A major biotinylated 90 kD protein was detected by immunoblot analysis with streptavidin-HRP (Fig. 4). Labeling of p90 with biotin-**1** was abolished by pretreatment of the cytosol 1 μM of unlabeled **1**. Thus, p90 is completely saturated by **1** at a concentration of 1 μM. Immunoprecipitation of Rsk2 with a monoclonal antibody (Santa Cruz Biotech) followed by immunoblot analysis with streptavidin-HRP

demonstrated unequivocally that biotin-1 irreversibly targets Rsk2 in Xenopus egg cytosol, and that binding is saturable by 1 μM of 1 (Fig. 15 and as shown below). Note that Xenopus and human Rsk2 are 92% identical.



Biotin-1 irreversibly targets Rsk2 in Xenopus egg cytosol.

[0057] Because streptavidin-HRP detection resulted in a high background, even in untreated cytosol, we prepared bodipy-1, which contains the green fluorophore FL-bodipy (Molecular Probes). Bodipy-1 labeled a single major protein of 90 kD (Fig. 18 and below), as detected by a confocal laser gel scanner (Amersham). Labeling was abolished by pretreatment with 1 μM of 1 or 2. Labeling of minor bands by bodipy-1 was not prevented by 1 or 2, suggesting that these proteins are nonspecifically targeted by bodipy-1.



Left panel: confocal laser gel scan reveals that bodipy-1 irreversibly targets Rsk2 in Xenopus egg cytosol. **Right panel**: Coomassie blue-stained gel reveals all cytosolic proteins.

[0058] Enoate 2 blocks Rsk autophosphorylation of Ser381, which is catalyzed by its cysteine-containing C-terminal kinase domain. The enoate does not block Erk phosphorylation. Swiss 3T3 cells were serum starved for 24 hours and treated with 5 μM enoate or 0.1% DMSO (control) for 1 hour. The cells were then stimulated with growth factors for 10 min. and lysed with 1x SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Phosphorylation-specific antibodies against Rsk and Erk were purchased from Cell Signaling. Antibodies against nonphosphorylated Rsk and Erk were purchased from Santa Cruz Biotech and Cell Signaling, respectively.

[0059] Compounds that inhibit the kinases according to this invention would be useful as anti-cancer and [or?] anti-inflammatory drugs and/or as immunosuppressants. In addition they also would be useful as inhibitors to probe the function of specific serine/threonine kinases in mammalian cells.

[0060] In one aspect the invention provides *in vitro, ex vivo,* and *in vivo* assays for inhibitors of the eleven kinases described herein, either as a group or individually. In particular, the assays can be used to test for compounds that possess this activity for testing for binding to or inhibition of the activity of the kinase or kinases in question. Typically in such assays, the compound or compounds to be tested are contacted with the kinase or kinases and suitable tests are carried out to ascertain whether the normal activity of the kinase(s) has been inhibited. For example, the results of the assay may be compared to the a control assay that comprises the kinase(s) alone, without the test compound(s), using any known activity of the kinase(s) as the comparison standard.

[0061] Binding of test compounds to kinases can be performed in solution, in a bilayer membrane, attached to a solid phase, in a lipid monolayer, or in vesicles. Binding of test compounds to the kinases can be tested by measuring or observing changes in kinase activity or by , *e.g.*, changes in spectroscopic characteristics or in chromatographic or solubility properties. Binding of test compounds can also be ascertained in competitive binding assays, for example, by ascertaining whether unlabeled test compounds prevent the interaction between the kinase and a biotinylated or fluorescent derivative of a reference compound. An example of such a reference compound is biotinylated compound 1:

[0062] The assays that form an aspect of this invention may be designed to screen large chemical libraries for inhibition of one or more of the kinascs using automated assay steps, which are typically run in parallel (*e.g.*, in microtiter formats on microtiter plates in robotic assays). In one preferred embodiment, high throughput screening methods are used that involve providing a combinatorial chemical or other library containing a large number of potential inhibitory compounds. Such libraries are then screened in one or more assays, as described herein, to identify those library members (either particular chemical species or subclasses) that display the desired activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

[0063] Thus, another aspect of this invention lies in libraries, such as combinatorial libraries, of compounds that are produced for testing based on activity, i.e., inhibition of one or more of the kinases described herein, within the general definitions of compounds, such as formulas (I) - (V). A combinatorial chemical library is a collection of such chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library is formed by combining a set of chemical building blocks in every possible way for a given compound type. Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[0064] Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

Other aspects of the invention involve the engineering or modification of protein kinases other than Rsk1-4, Msk1-2, Plk1-3, MEKK1, and Nek2 so as to render them susceptible to inhibition, for instance, by the compounds described herein.

Thus, this invention also involves the engineering or modification of a protein kinase by replacing a valine residue within the ATP binding site of the protein kinase with a cysteine residue. This can render the enyzmatic activity of the modified protein kinase susceptible to inhibition by the compounds disclosed herein.

Alternatively, this invention involves the engineering or modification of a protein kinase that already contains a cysteine in the ATP-binding site corresponding to Cys436 of Rsk2. These kinases include Rsk3, Msk1-2, Plk1-3, MEKK1, and Nek2. The engineering or modification

of the protein kinase is achieved by replacing a methionine, leucine, isoleucine, lysine, arginine, tryptophan, glutamine, asparagine, proline, tyrosine, histidine, glutamic acid, aspartic acid, valine, or phenylalanine residue in the gatekeeper position of the ATP binding site with a smaller residue, e.g. a threonine, serine, alanine, or glycine residue. This can render the kinase susceptible to inhibition by some compounds of this invention, and can serve to identify such compounds.

[0065] Selective inhibitors of individual kinases are useful as tools for elucidating kinase function in signaling networks; however, it is difficult to find such inhibitors due to the highly conserved nature of the kinase catalytic domain. However, engineering or modification of a protein kinase can transform a kinase that has no known inhibitors (or that can be only inhibited reversibly) into one that for the first time can be inhibited (or can be inhibited irreversibly), for example by compounds of this invention . The now modified kinase can be used to elucidate kinase functioning in signaling networks, for example by being introduces into genetically transformed animals.

Chemical genetic strategies have been successfully used to generate highly selective inhibitors for a single engineered kinase. The aspects of this invention that relate to engineering or modifying kinases involves a new chemical genetic approach that relies on the design of active site-directed, electrophilic tyrosine kinase inhibitors. The method involves replacing a conserved valine in the active site of selected Src tyrosine kinases with a nucleophilic cysteine residue. Electrophilic variants of the Src kinase inhibitor PP1 (which does not contain an electrophile) were synthesized by appending halomethylketone substituents to the C6 position of a pyrrolo[2,3-*d*]pyrimidine scaffold. The inhibitors selectively and irreversibly inactivated the engineered kinases *in vitro* and *in vivo*. This chemical genetic strategy is expected to be broadly applicable to other members of the kinase superfamily.

The transfer of the γ-phosphate group of ATP to specific tyrosine, threonine, or serine residues on protein substrates is a major regulatory mechanism for controlling enzymatic activity and protein-protein interactions in eukaryotic cells. Protein kinases, the enzymes that catalyze this critical reaction, comprise 1.7% of the human genome. As these enzymes play a significant role in controlling cell proliferation and migration, their overexpression or misregulation can have a causative role in human diseases, such as cancer. The development of cell-permeable small molecules that rapidly inactivate kinases has contributed to our

understanding of their functions in cell regulation, and has led to the discovery of novel therapeutic targets. However, it has been difficult to design selective inhibitors that target individual kinases because their active sites are so structurally similar.

To circumvent this problem, Shokat and coworkers have developed a chemical genetic approach for generating selective inhibitors of individual kinases. The method involves mutating a bulky, usually hydrophobic, residue within the ATP-binding site to either glycine or alanine. This creates an enlarged pocket that can uniquely accept a chemically modified inhibitor that is too large to fit in the active site of any wild-type kinase. This approach was first applied to the Src family of nonreceptor tyrosine kinases, but has been extended to receptor tyrosine kinases as well as serine/threonine kinases. While many of the resulting glycine mutants have wild-type levels kinase activity, there are some cases in which kinase activity is severely diminished. For example, mutation of the methionine residue in the active site of either Jak2 or Syk2 to glycine leads to almost complete loss of kinase activity. Therefore, complementary strategies for the selective inhibition of kinases are needed.

According to the present invention, selective inhibition of individual kinases is achieved using an alternative chemical genetic approach. This method involves engineering a suitably poised nucleophile in the ATP-binding site to react with an electrophilic inhibitor, thus promoting covalent inactivation of the kinase. Belshaw and co-workers recently reported a conceptually similar approach in which an acrylamide-substituted cyclosporin derivative irreversibly inhibited an engineered cysteine mutant of the prolyl isomerase cyclophilin.

Electrophilic inhibitors, as in the compounds of this invention, are advantageous over reversible inhibitors for two reasons. First, because the dissociation rate is zero, the concentrations needed to block protein function are potentially much lower than reversible inhibitors. This is especially true for protein kinase inhibitors, which almost always bind competitively with respect to ATP (present in the cytoplasm at millimolar concentrations). Second, electrophilic inhibitors have an additional element of specificity that is dependent on covalent bond formation. For example, the acrylamide-substituted quinazoline, PD 168393, was shown to specifically alkylate a cysteine residue in the ATP-binding site of the epidermal growth factor receptor. PD 168393 was far superior to reversible quinazoline inhibitors in an animal carcinoma model.

In the beginning of our work we designed and synthesized electrophilic variants of the Src kinase inhibitor PP1, which, as expected, reacted with an engineered cysteine residue in the

ATP-binding site of Src-family tyrosine kinases. The novel compounds of our invention potently inactivated the engineered kinases, yet were poor inhibitors of wild-type (wt) enzymes. Importantly, a chloromethylketone (cmk) derivative (4) irreversibly blocked the function of mutant v-Src in mammalian cells, yet had no effect on wt v-Src .

Recently, the crystal structure of Hck (a Src-family kinase) in complex with PP1 was determined. This structure shows PP1 bound within the ATP-binding site of the kinase, with the tolyl substituent inserted into an adjacent hydrophobic pocket (Fig. 20B). Based on this structure, we searched for residues in the active site that made van der Waals contacts with the N6 position of PP1 (Fig. 20A) and selected valine 281 to mutate to cysteine. Val281 is adjacent to a flexible, glycine-rich loop. Thus it is more likely that a cysteine at this position would be properly oriented for nucleophilic attack. Electrophilic variants of the Src kinase inhibitor PP1 were designed. These compounds had an electrophile-bearing carbon in place of the N6 of PP1 (Fig. 20A). Fig. 20B shows a model of a chloromethylketone inhibitor bound to the cysteine-containing Hck mutant. The wild-type kinase does not contain any cysteines within the active site, but the new electrophiles of our invention specifically inactivate the mutant kinase via covalent bond formation.

Chemical Synthesis of halomethylketone derivatives is described in Figure 21. First, 1 was synthesized in overall 48% yield from commercially available materials (see Supporting Information). The hydroxypropyl substituent at N7 was chosen so that an affinity tag (e.g. biotin) could be attached to this position.

Halomethylketones **3-5** were synthesized as illustrated in Figure 21. Referring to that Figure, the synthesis commences with the selective bromination of the 6-position of **1** with NBS (80% yield). Conversion of 6-bromo **1** to enol ether **2** was accomplished in 82% yield by palladium catalyzed Stille coupling with α-(ethoxy vinyl)tributyl tin. Enol ether **2** served as a key intermediate from which compounds **3-5** could be synthesized in two or three steps. Bromomethylketone (bmk) **3** was synthesized in 60% yield via bromination of **2** with NBS at $-20\ ^{0}C$, followed by removal of the TBS protecting group with 1N HBr. Because of the electron donating effects of the nitrogen in the pyrrole ring of **2**, we reasoned that chloromethylketone (cmk) **4** could be synthesized directly from **2** with the less reactive electrophilic halogenating reagent, NCS. Indeed, enol ether **2** could be converted to cmk **4** with NCS, albeit in modest yield (40% overall), following TBS removal with 1N HCl.

Nucleophilic fluorination of 3 with KF and subsequent TBS removal furnished fluoromethylketone (fmk) 5 in 42% overall yield.

Time-dependent inhibition of V282C Fyn kinase activity *in vitro*. Halomethylketones 3-5 were screened against the catalytic domain of wt Fyn and V282C Fyn, which were bacterially expressed and purified as a glutathione-S-transferase (GST) fusion proteins. All three compounds exhibited time-dependent inactivation of V282C Fyn kinase activity. At a concentration of 10 nM cmk 3 or bmk 4, complete inactivation was achieved within 30 min (see Figure 22), whereas inactivation by fmk 5 was much slower (data not shown). Significantly, 3-5 do not affect the kinase activity of wt Fyn at concentrations required to inhibit 90% of the activity of V282C Fyn. These data are consistent with the notion that compounds 3-5 selectively inactivate V282C Fyn via covalent modification of the engineered cysteine residue.

Covalent labeling and selective inhibition of v-Src-es1 in mammalian cells. To test whether cmk 4 could covalently label and inhibit Src kinases *in vivo*, we generated NIH3T3 fibroblast cell lines that stably overexpress either a wt v-Src allele or an electrophile-sensitive mutant, V281C v-Src (v-Src-es1). v-Src is constitutively active, and as a result of its expression in these cells, there is a substantial increase in phosphotyrosine levels. Because Src and Fyn are 85% identical in their kinase domains, we expected that an active site cysteine mutant of v-Src would be sensitive to cmk 4 with similar potency to that observed for V282C Fyn. To test whether cmk 4 forms an irreversible, covalent bond with V281C v-Src, we conjugated biotin to the hydroxypropyl substituent (Fig. 23A). This point of attachment was chosen based on the model of cmk 4 bound to Hck in which the hydroxypropyl substituent is pointing out into solution.

NIH3T3 fibroblasts expressing either v-Src-es1 or v-Src were pretreated with increasing concentrations of cmk 4. Whole cells lysates were then prepared and treated with biotin-cmk (2 μM). Immunoprecipitation of v-Src with a monoclonal antibody followed by immunoblot analysis with streptavidin (horseradish peroxidase conjugate) demonstrated that biotin-cmk covalently targeted v-Src-es1 (Fig. 23B). Labeling of v-Src-es1 was abolished by pretreatment of intact cells with 1 μM cmk 4 (IC_{50} < 100 nM). The absence of covalent labeling observed for wt v-Src demonstrates that the engineered cysteine is required for modification by cmk 4 (Fig. 23B).

We then investigated whether cmk 4 could block the function of v-Src-es1 in mammalian cells by monitoring global phosphotyrosine levels in cells expressing either wt v-Src or v-Src-es1. Cells overexpressing v-Src-es1 showed a dose-dependent decrease in tyrosine phosphorylation upon treatment with cmk 4 (Fig. 24), with a near-complete reduction of detectable phosphotyrosine at a concentration of 1 μM. In contrast, cmk 4 at 1 μM had no apparent effect on cells overexpressing wt v-Src. These data are consistent with the labeling experiments and indicate that the specificity of cmk 4 for v-Src-es1 is due to the selective covalent modification of a single cysteine within the active site of the kinase.

Reversion of transformed morphology in cells expressing v-Src-es1. Overexpression of v-Src in NIH3T3 fibroblasts causes morphological transformation. Nontransformed NIH3T3 cells have a flat morphology, characterized by long bundles of filamentous actin ("stress fibers") that can be visualized by fluorescence microscopy. In contrast, NIH3T3 cells overexpressing wt v-Src or v-Src-es1 have a disorganized actin cytoskeleton that results in a round morphology. Treatment of cells expressing v-Src-es1 with cmk 4 (1 μM) for 16 h caused reversion of the transformed morphology as evidenced by the appearance of actin stress fibers after fixing the cells and staining filamentous actin with FITC-phallodin (Fig. 25A). Under the same conditions, cells expressing wt v-Src are not affected by cmk 4 and maintain a round morphology (Fig. 25A). The effects of cmk 4 on cell morphology were quantified by counting 100-200 cells under each treatment condition for both wt v-Src and v-Src-es1. Approximately 80% of the cells expressing v-Src-es1 regain a flattened morphology upon treatment with 1 μM cmk 4, whereas less than 2% of cells expressing wt v-Src have actin stress fibers (Fig. 25B). These data show that treatment of cells expressing v-Src-es1 with 1 μM cmk 4 is sufficient to block the ability of v-Src to cause morphological transformation.

Formulation and administration.

[0066] Compounds identified as active kinase inhibitors can be administered to a patient at therapeutically effective doses to prevent, treat, or control conditions, for example to act as immunosuppressive or anti-inflammatory agents. Compositions containing the substances are administered to a patient in an amount sufficient to elicit an effective therapeutic response in the patient. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "therapeutically effective amount". The dose or amount will be determined by the efficacy of the particular active substance employed and the condition of

the subject. The size of the dose also will be determined by the existence, nature, and extent of any adverse effects that accompany the administration of a particular compound in a particular subject.

[0067] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio, LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue to minimize potential damage to normal cells and thereby reduce side effects.

[0068] The data obtained from cell culture assays and animal studies can be used to formulate a dosage range for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration. For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography (HPLC).

[0069] Pharmaceutical compositions for use in the present invention can be formulated by standard techniques using one or more physiologically acceptable carriers or excipients. The compounds and their physiologically acceptable salts and solvates can be formulated for administration by any suitable route, including via inhalation, topically, nasally, orally, or parenterally (*e.g.*, intravenously, intraperitoneally, intravesically or intrathecally).

[0070] For oral administration, the pharmaceutical compositions can take the form of, for example, lozenges, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients, including binding agents, for example, pregelatinized cornstarch, polyvinylpyrrolidone, or hydroxypropyl methylcellulose; fillers, for example, lactose,

microcrystalline cellulose, or calcium hydrogen phosphate; lubricants, for example, magnesium stearate, talc, or silica; disintegrants, for example, potato starch or sodium starch glycolate; or wetting agents, for example, sodium lauryl sulfate. Tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives, for example, suspending agents, for example, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats; emulsifying agents, for example, lecithin or acacia; non-aqueous vehicles, for example, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils; and preservatives, for example, methyl or propyl-p-hydroxybenzoates or sorbic acid. The preparations can also contain buffer salts, flavoring, coloring, and/or sweetening agents as appropriate. If desired, preparations for oral administration can be suitably formulated to give controlled release of the active compound.

[0071] For administration by inhalation, the compounds may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized acrosol, the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base, for example, lactose or starch.

[0072] The compounds can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents, for example, suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

[0073] Furthermore, the compounds can be formulated as a depot preparation. Such long-acting formulations can be administered by implantation (for example, subcutaneously or

intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0074] The compositions may also be in the form of controlled release or sustained release compositions as known in the art, for instance, in matrices of biodegradable or non-biodegradable injectable polymeric microspheres or microcapsules, in liposomes, in emulsions, and the like.

[0075] The compositions can, if desired, be presented in a pack or dispenser device that can contain one or more unit dosage forms containing the active ingredient. The pack can, for example, comprise metal or plastic foil, for example, a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

[0076] Depending on their chemical and physical nature, kinase-inhibitory compounds may be included in the compositions and administered to the patient per se, or in another form such as a salt, solvate, complex, chelate or other derivative as appropriate or as needed for good formulation or administration of the substance. Likewise, a prodrug of the substance may be included in the compositions, that is, a substance that releases the active substance either on preparation of the composition or on administration of the composition to the patient or subject.

[0077] In carrying out the invention, a single inhibitory compound, or a combination of compounds according to this invention, i.e. that interact with cell adhesion receptors, may be administered to a patient. The effective compounds may be administered alone or in combination with (or in time proximity to) other therapeutic agents administered for similar or other therapeutic purposes, for example administration of a compound according to this invention together with an adjuvant or other anti-inflammatory agent. Similarly, compositions containing one or more of the compounds of this invention may also contain other therapeutic agents.

[0078] The present invention also includes arrays for testing substances for interaction with or binding to the kinases. Typically such arrays will be used for testing combinatorial or other libraries. The arrays will comprise standard equipment such as a plate, which will contain kinases arranged on the surface of the plate, for example in wells or bound to certain

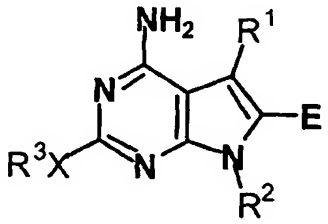
locations on the surface. A plate or array may contain kinases of a single type or it may contain different kinases, located in prearranged fashion.

[0079] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A compound having a heterocyclic core structure comprised of two or more fused rings containing at least one nitrogen ring atom, and an electrophilic substituent that is capable of reacting with a cysteine residue within the ATP binding site of a kinase.

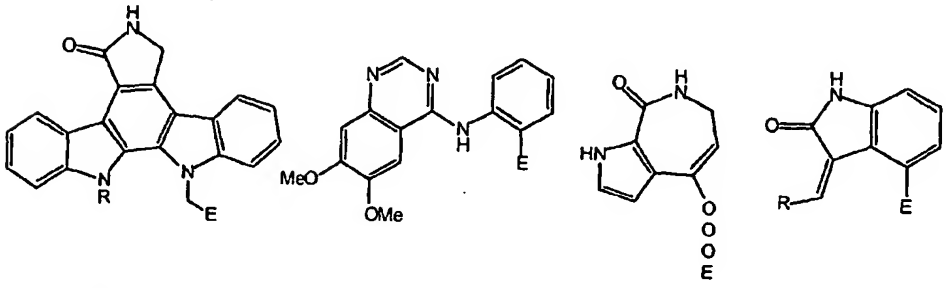
2. A compound according to claim 1 having the formula (I)



(I)

in which R^1 is an optionally substituted aromatic or heteroaromatic group; E is an electrophilic group; R^2 is an aliphatic, aromatic, or hetcroaromatic group optionally substituted with one or more polar groups; and R^3 X is hydrogen or an alkyl- or aryl-substituted ether, thioether, or amine.

3. A compound according to claim 1 having the formula (II), (III), (IV) or (V):



(II) (III) (IV) (V)

in which E is an electrophile and R is hydrogen or an optionally substituted aliphatic group.

4. A method of inhibiting a protein kinase that has one or more cysteine residues within its ATP binding site, comprising contacting the kinase with an inhibitory-effective amount of a compound according to claim 1.

5. A method of imparting to a protein kinase the capability of being inhibited by a compound according to claim 1 comprising replacing a valine residue within the ATP binding site of the protein kinase with a cysteine residue.

6. A method of imparting to a protein kinase the capability of being inhibited by a compound according to claim 1 comprising replacing a methionine, leucine, isoleucine, lysine, arginine, tryptophan, glutamine, asparagine, proline, tyrosine, histidine, glutamic acid, aspartic acid, valine, or phenylalanine residue in the gatekeeper position of the ATP binding site with a smaller residue.

Pyrrolopyrimidine as a scaffold for electrophilic "ATP mimetics"

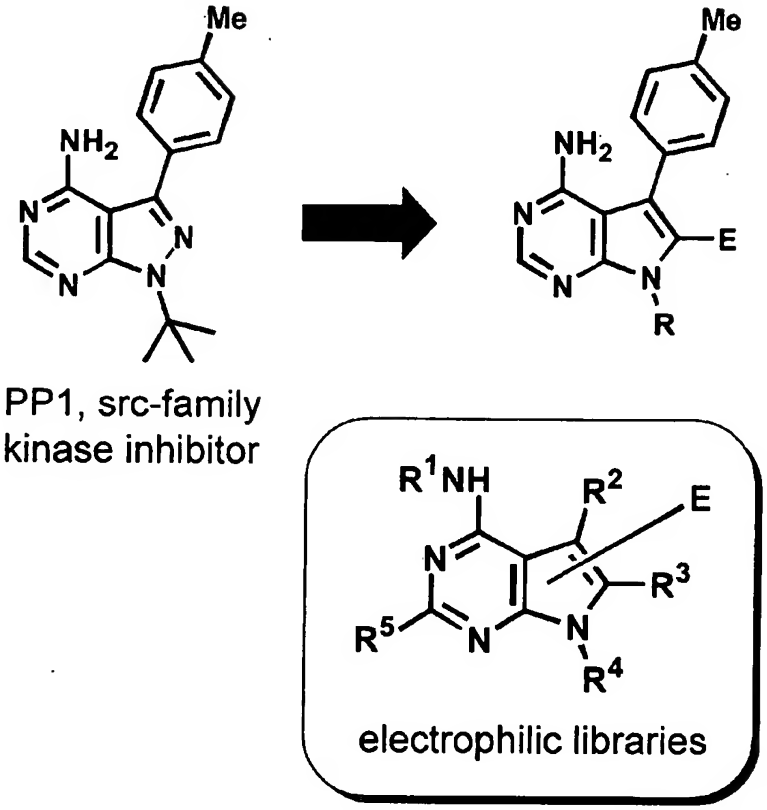


FIG. 1

PP1 bound to autoinhibited Hck

FIG. 2

The catalytic Lysadopts a different conformation in the activated kinase.

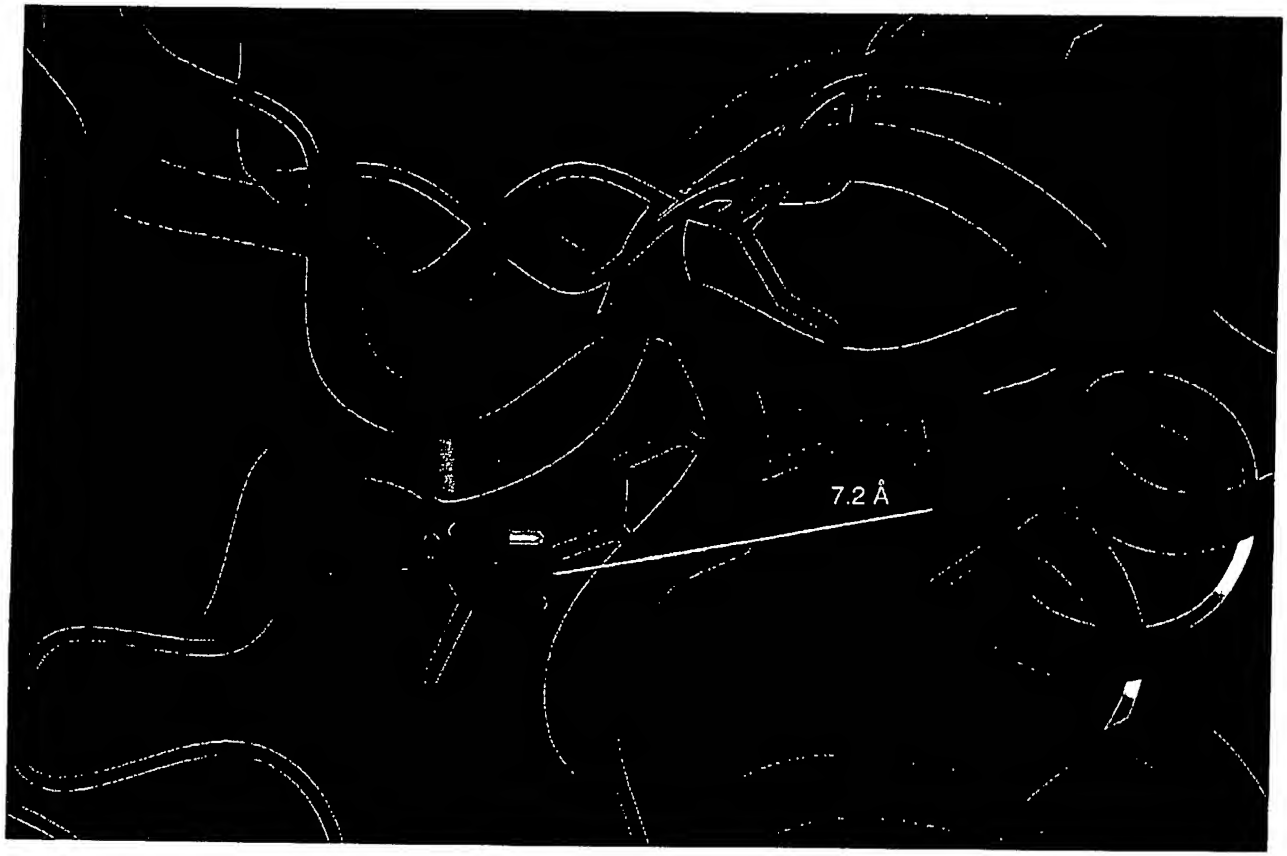


FIG. 3

An acid fluoride trap for the catalytic lysine

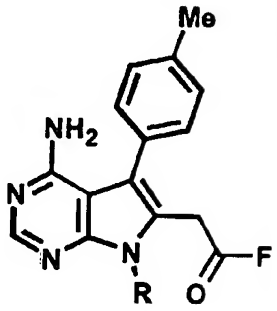


FIG. 4

Synthesis of the pyrrolopyrimidine scaffold

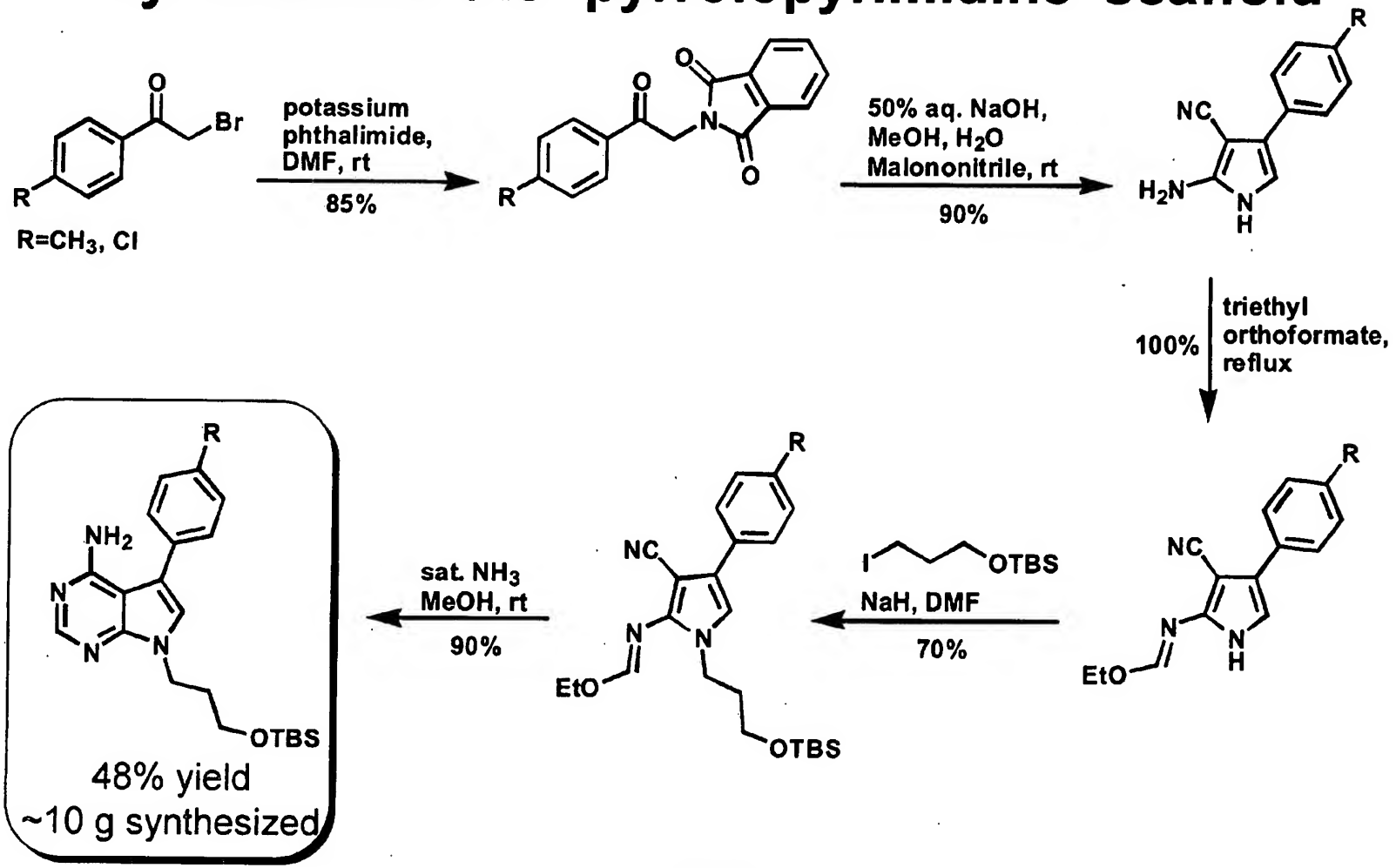


FIG. 5

Palladium rocks the house, yo!

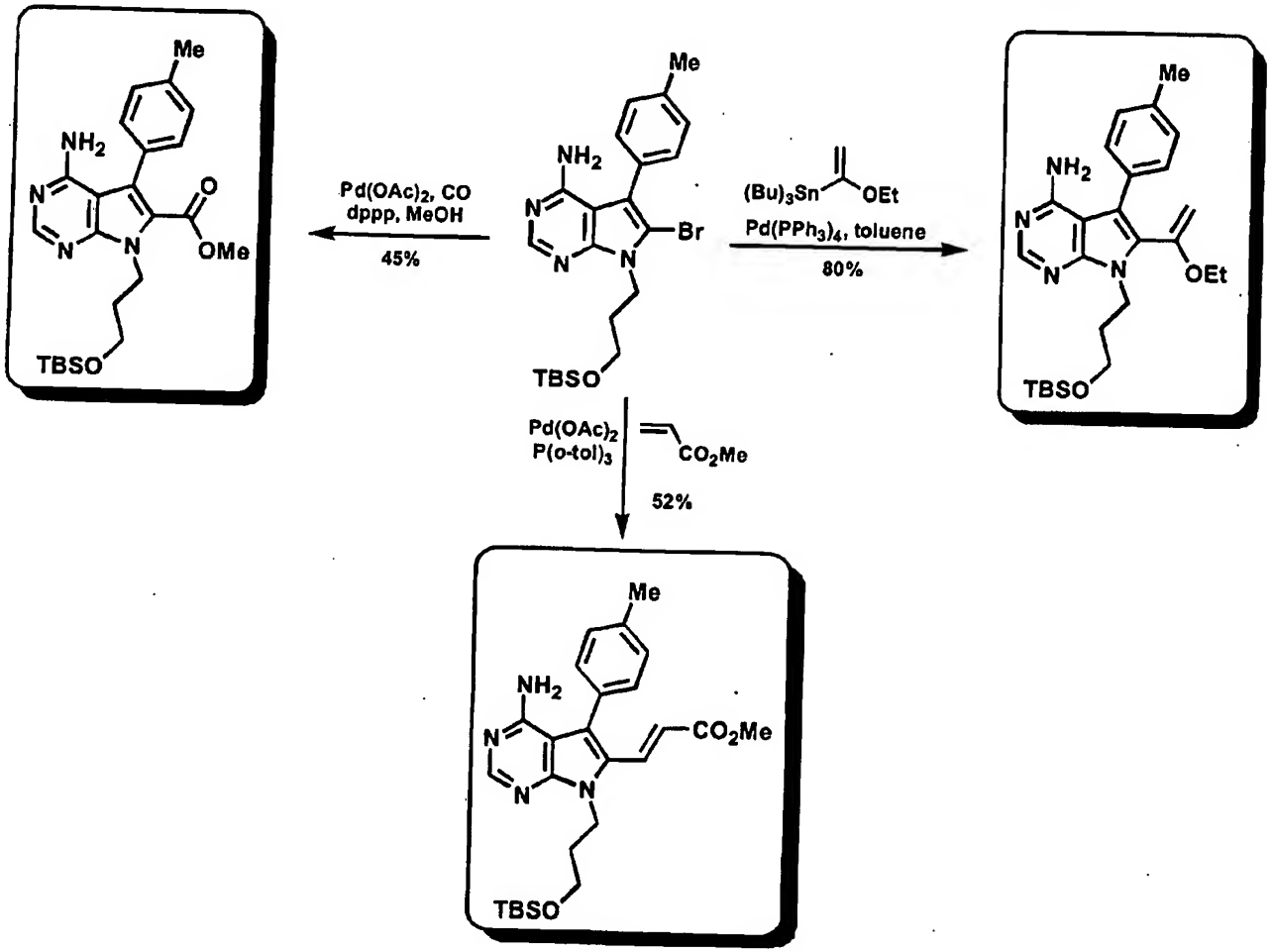


FIG. 6

Synthesis of α-haloketones

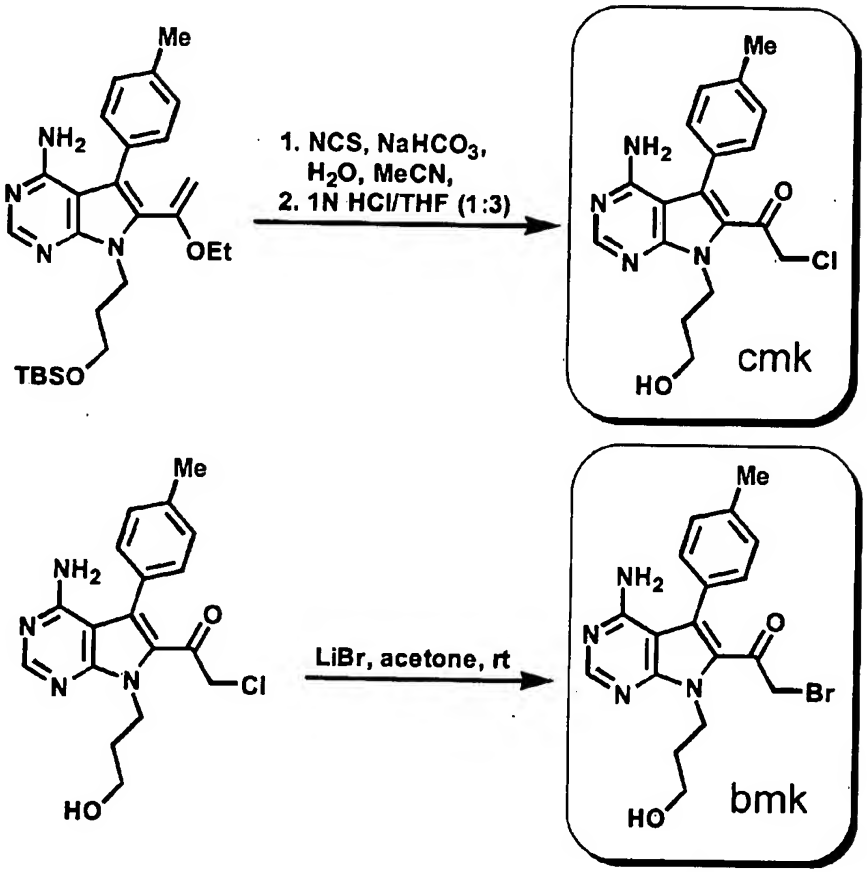


FIG. 7

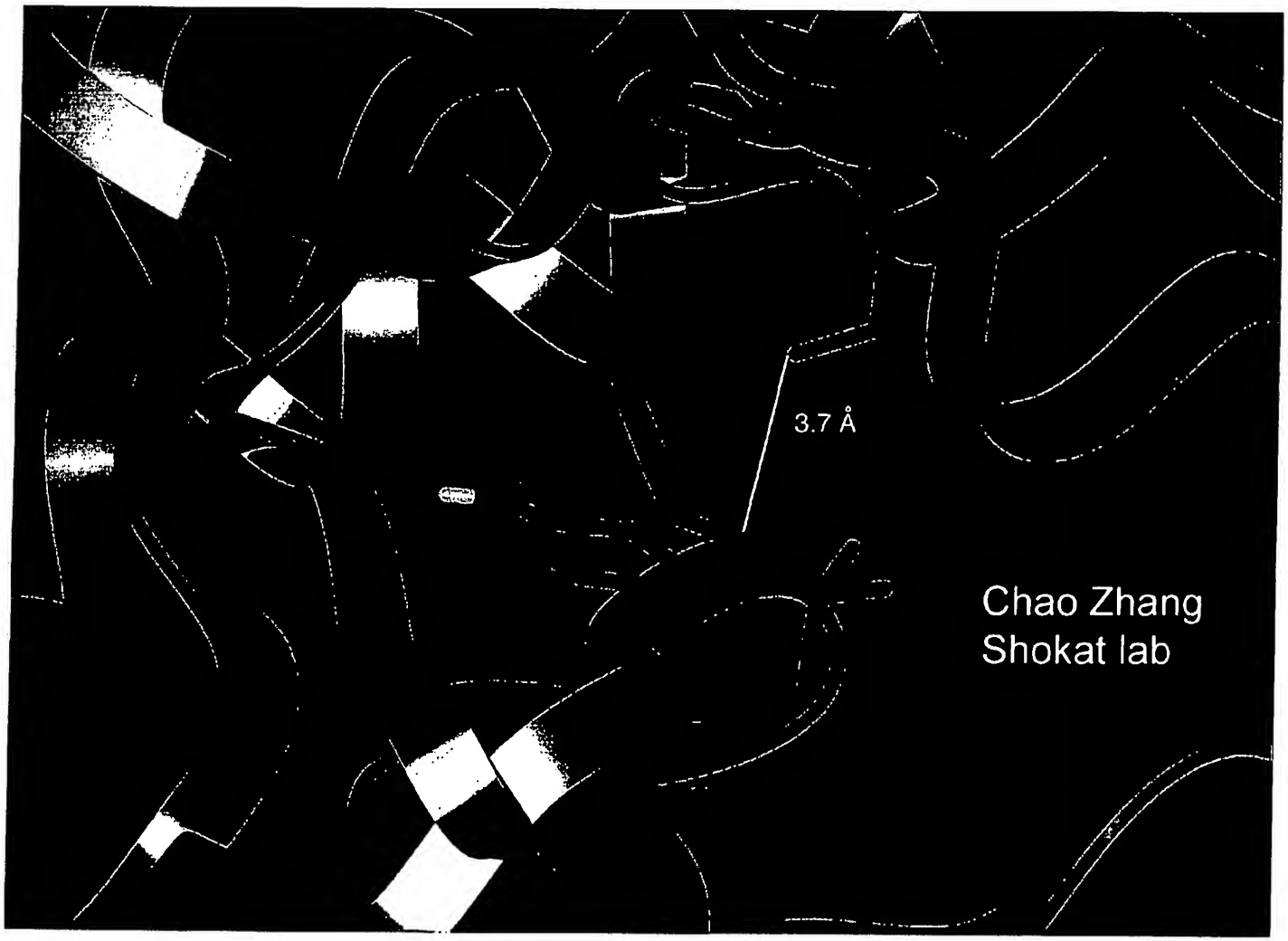


FIG. 8

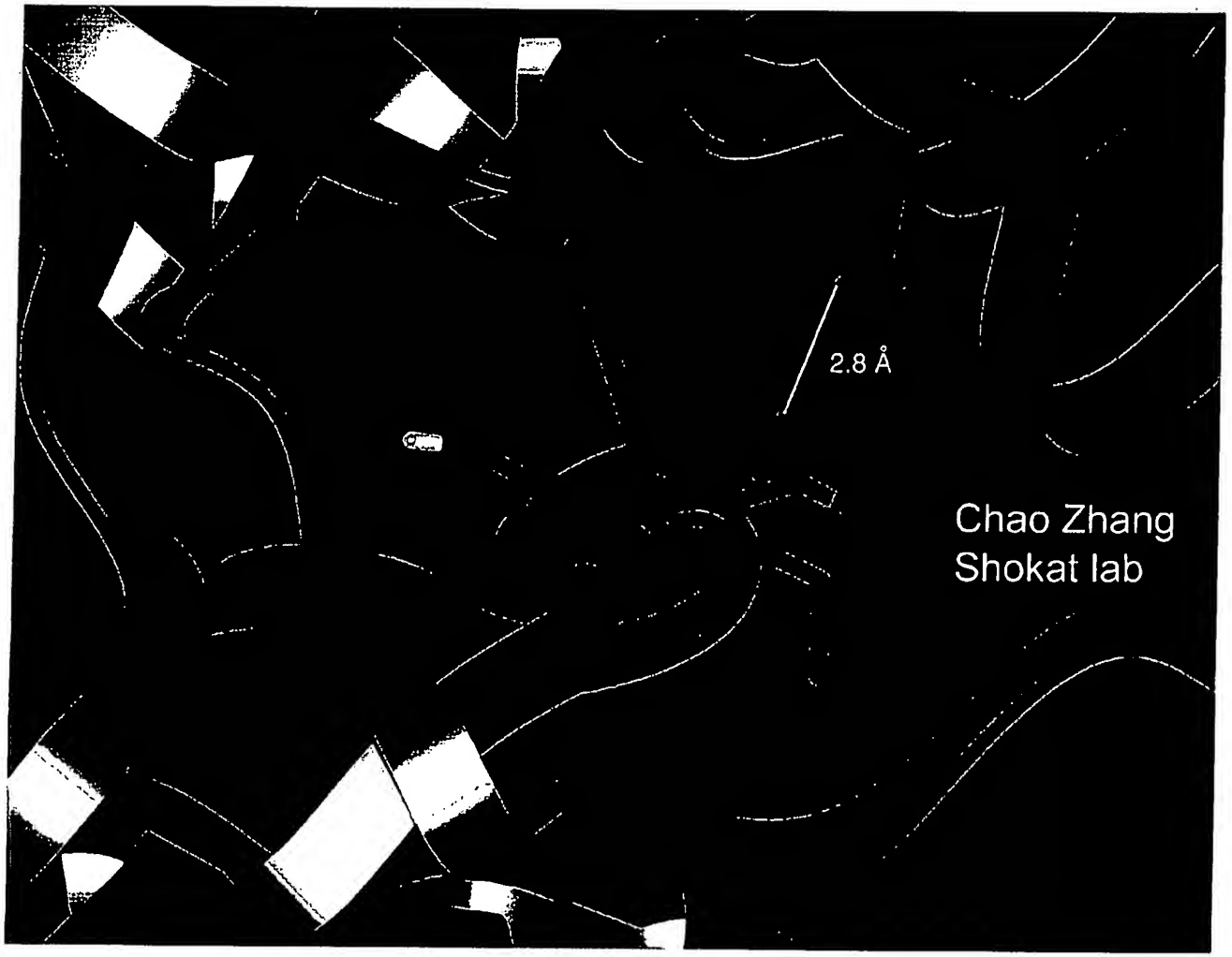


FIG. 9

Time-dependent inhibition of FynV282C

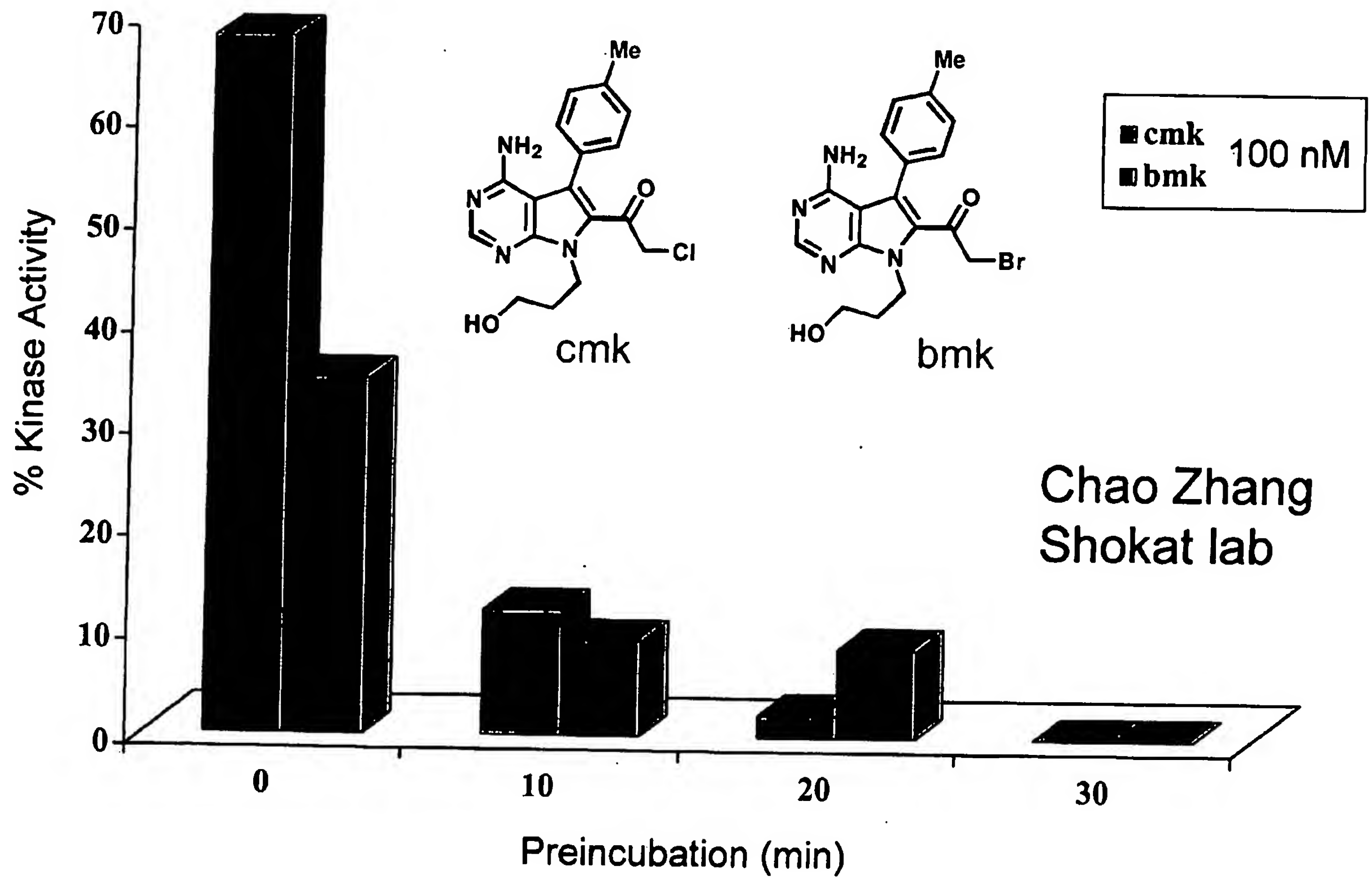


FIG. 10

Five (and only five) kinase subfamilies have an appropriately positioned Cys.

```
src      -------LRLEVK-LGQGCFGEVWMGTWNG--TTRVAIKTLKPGTMS---
rsk2     -IQFTDGYEVKED-IGVGSYSVCKRCIHKATN-MEFAVKIIDKS------
nek2     MPSRVEDYEVLHS-IGTGSYGRCQKIRRKSDG-KILVWKELDYGSMT---
mekk1    --REDTEWLKGQQ-IGLGAFSSCYQAQDVGTG-TLMAVKQVTYVRNTSSE
msk1     ----HYDLDLKDKPLGEGSFSICRKCVHKKSN-QALQVKIISKR------
plk      -----RRRYVRGRFLGKGGFAKCFEISDADTK-EVFAGKIVPKSLLLK--

src      ----PEAFLQEAQVMK--KLRHEKLVQLYAVVSEEP---IYIVTEYM
rsk2     ----KRDPTEEIEILLR-YGQHPNIITLKDVYDDGKY--VYVVTELM
nek2     -EVEKQMLVSEVNLLR--ELKHPNIVRYYDRIIDRTNTTLYIVMEYC
mekk1    QEEVVEALREEIRMMS--HLNHPNIIRMLGATCEKSN--YNLFIEWM
msk1     ---MEANTQKEITALK-LCEGHPNIVKLHEVFHDQLH--TFLVMELL
plk      -PHQREKMSMEISIHR--SLAHQHVVGFHGFFEDNDF--VFVVLELC
                                                "gatekeeper"
```

Only one of them has a Thr in the "gatekeeper" position.

Sasha Buzko, gatekeeper of the KSD

FIG. 11

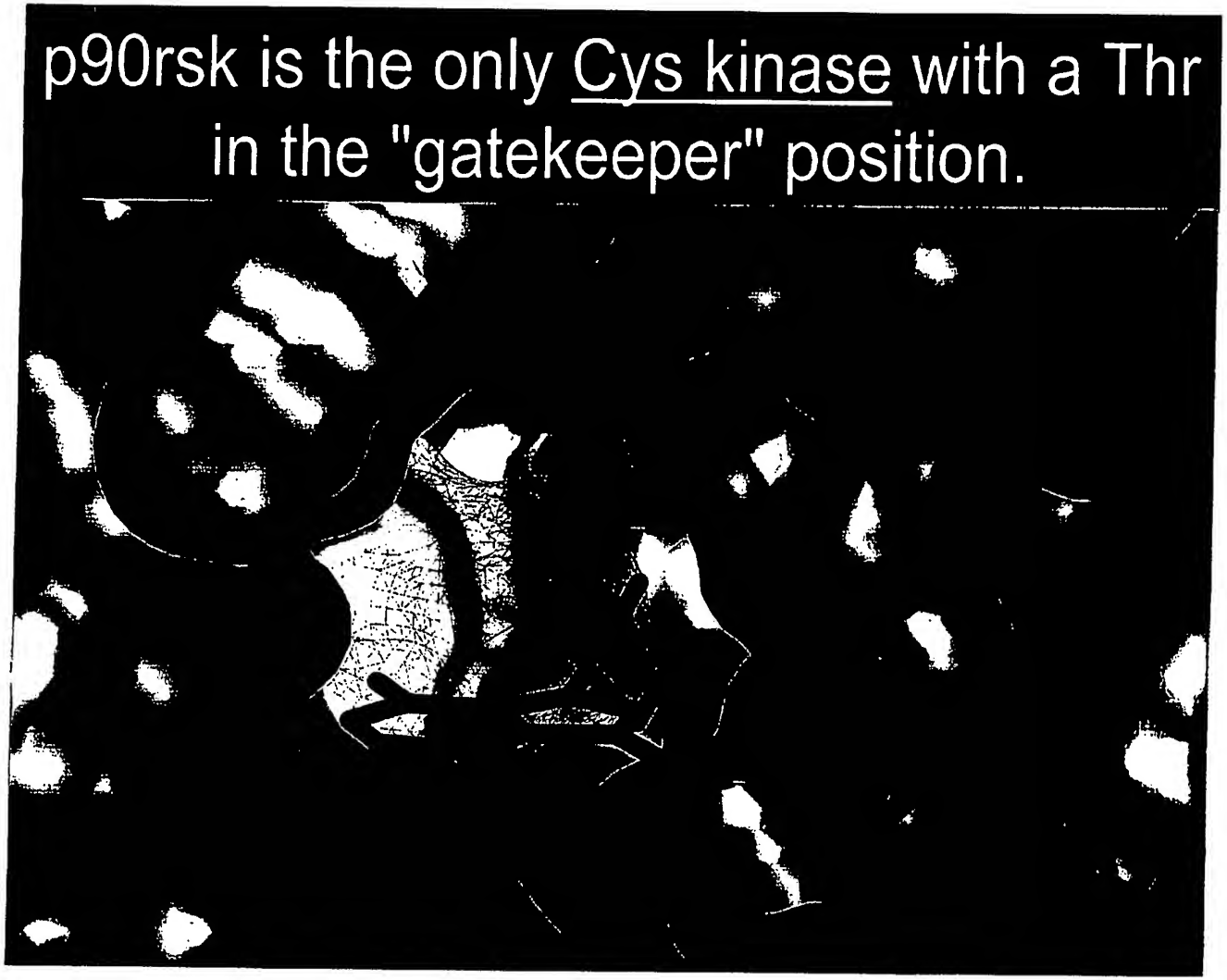


FIG. 12

Baroque regulation of p90rsk activity

Phosphorylation of a PDK1 docking site by the CTK is required for activation of the NTK.

The CTK is a Cys kinase.

p90rsk is a major downstream target of ras.

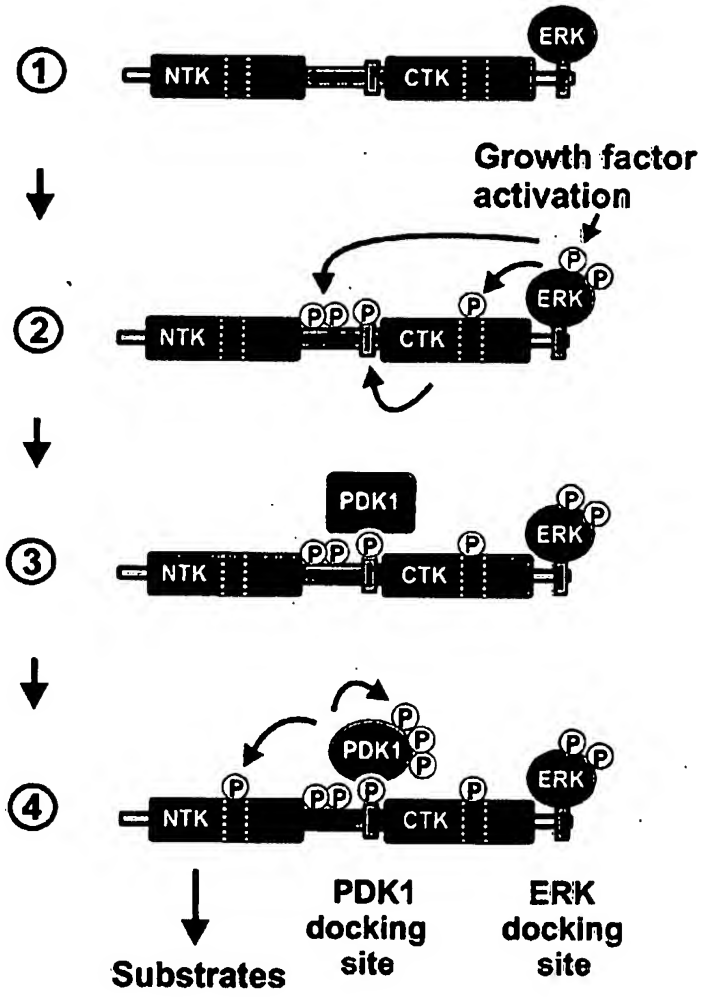
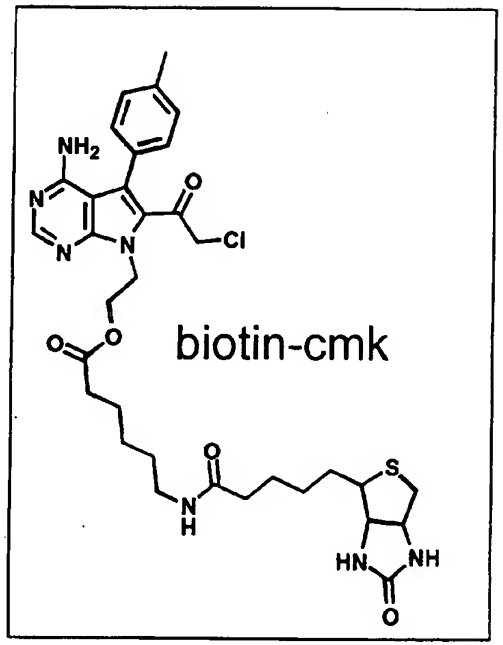
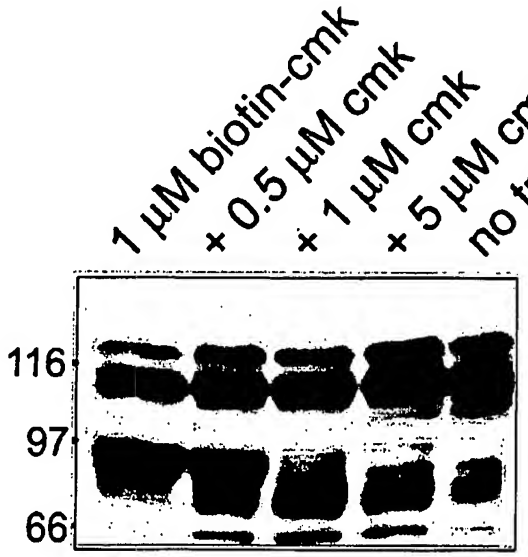


FIG. 13

Covalent modification of p90rsk in Xenopus egg extracts

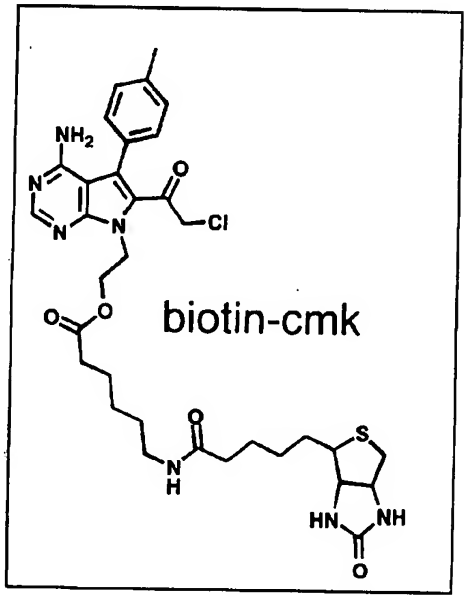




streptavidin blot
(crude extract)

FIG. 14

Covalent modification of p90rsk in Xenopus egg extracts



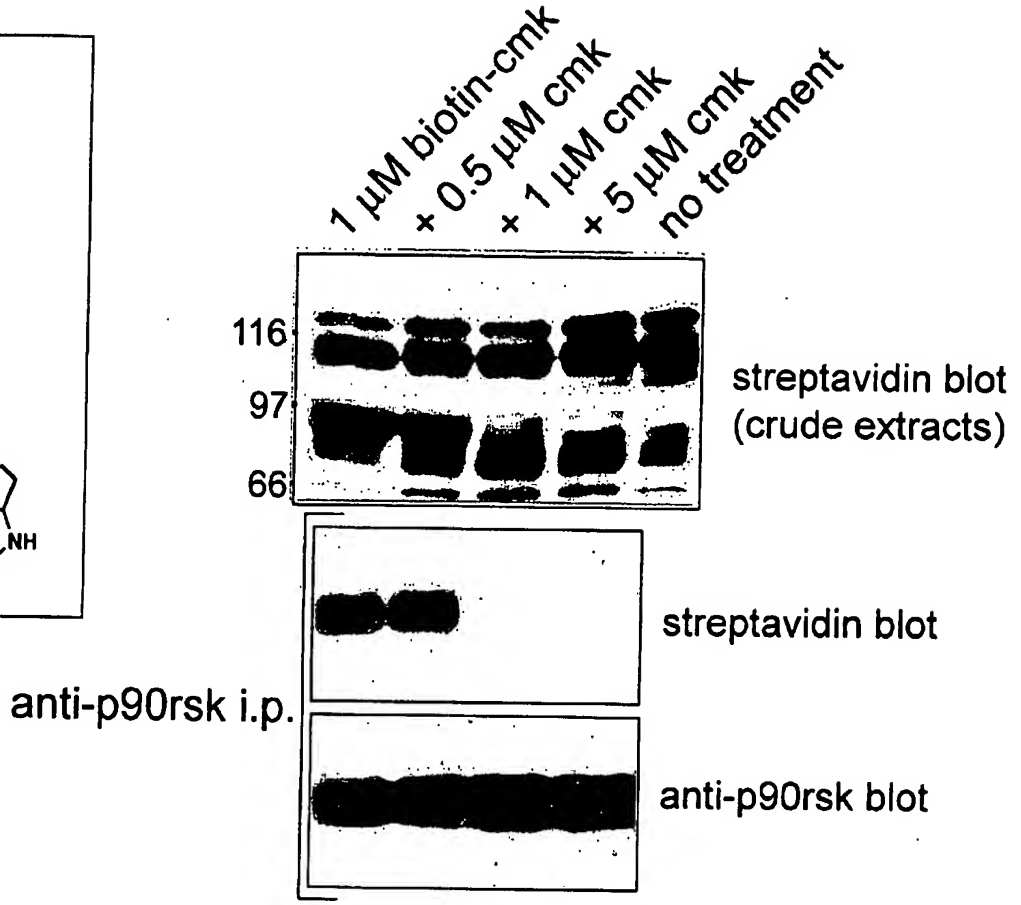


FIG. 15

Preparation of fluorescent cmk derivatives

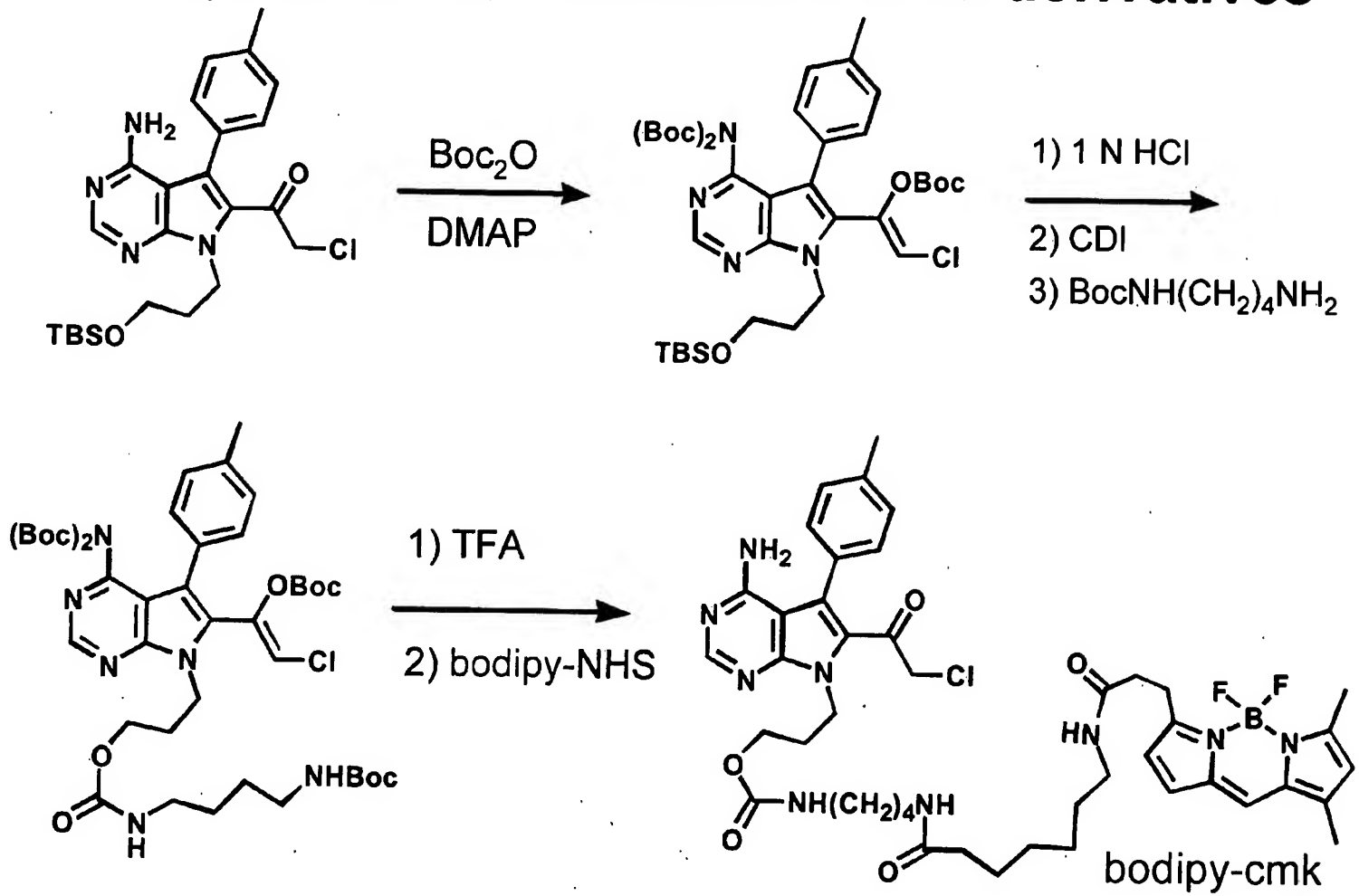


FIG. 16

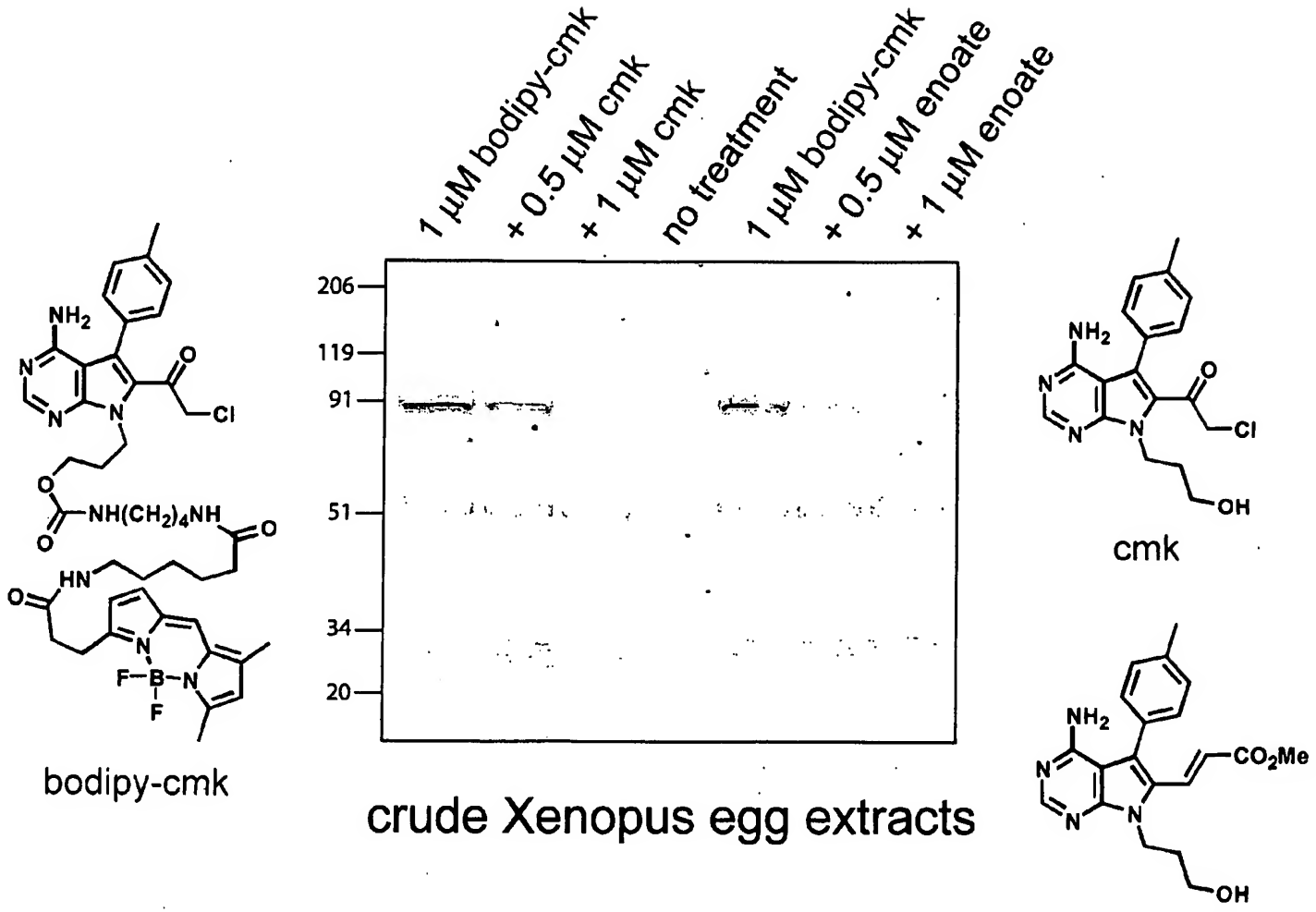


FIG. 17

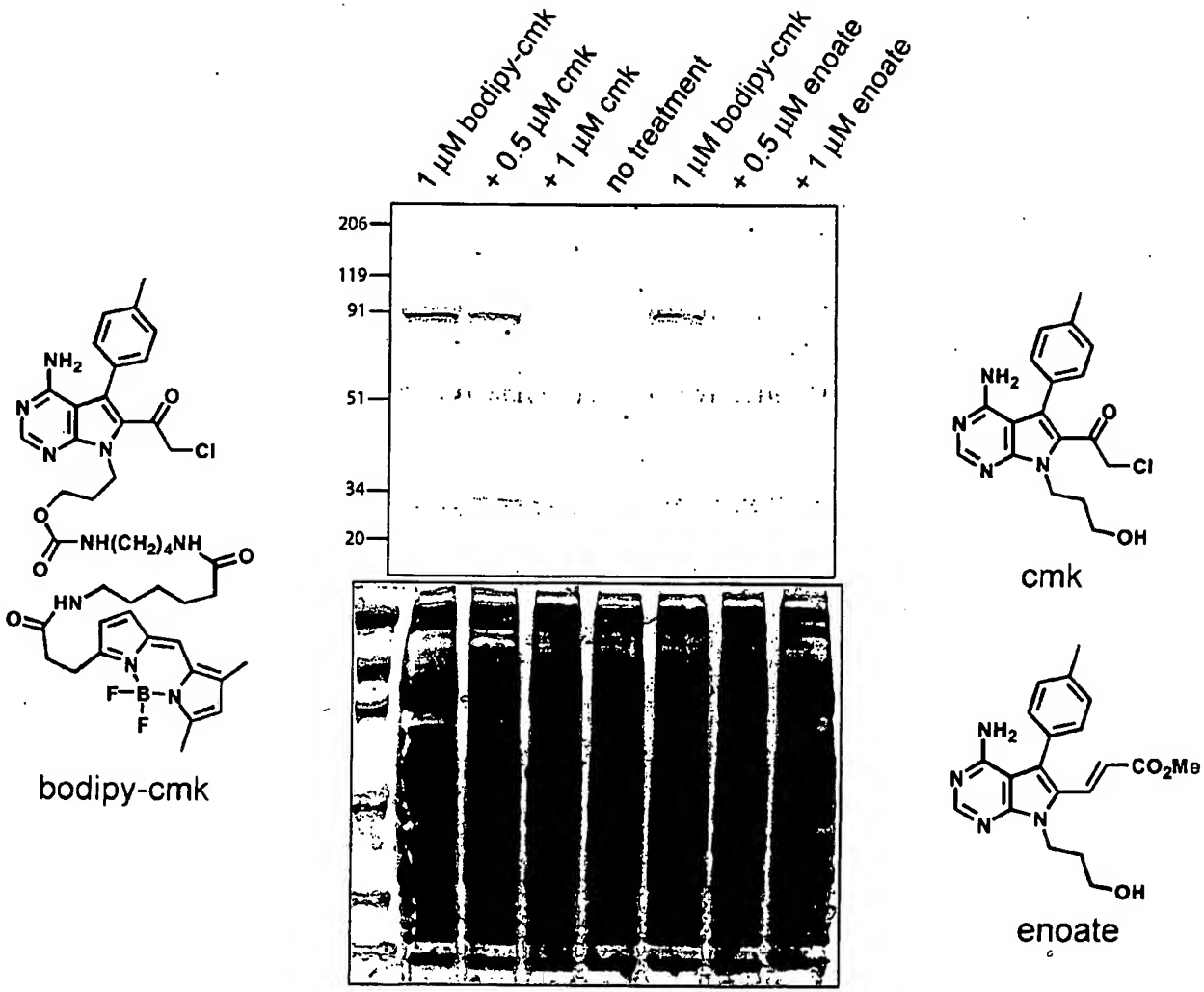


FIG. 18

Inhibition of Rsk autophosphorylation in Swiss 3T3 fibroblasts

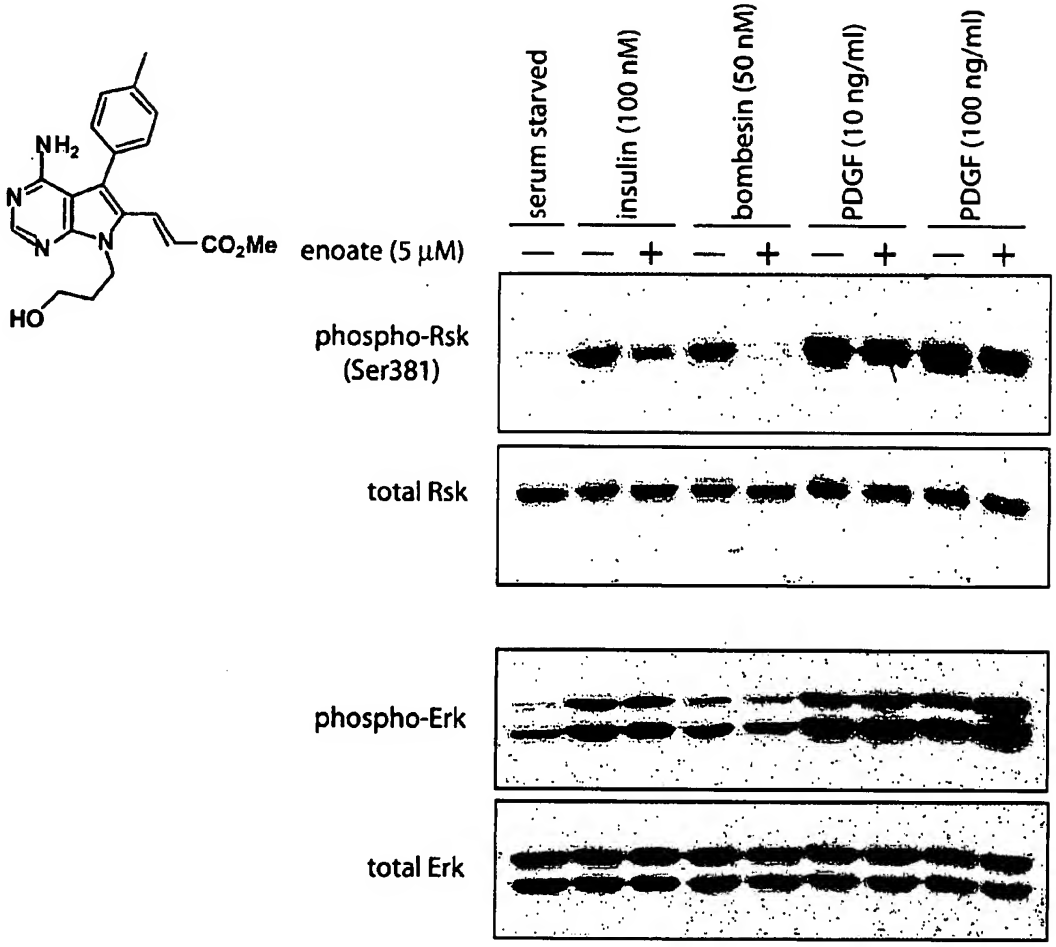
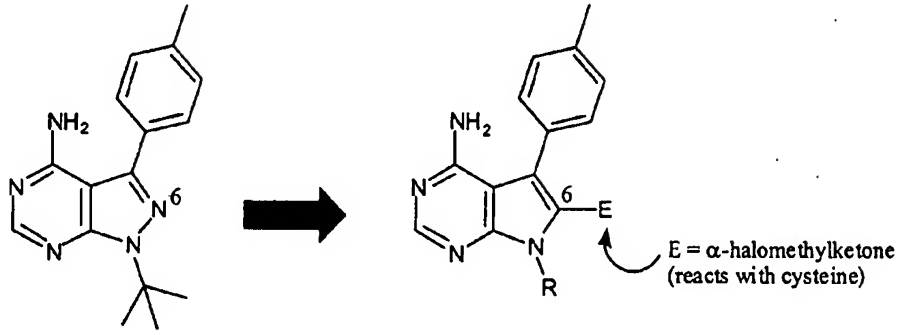


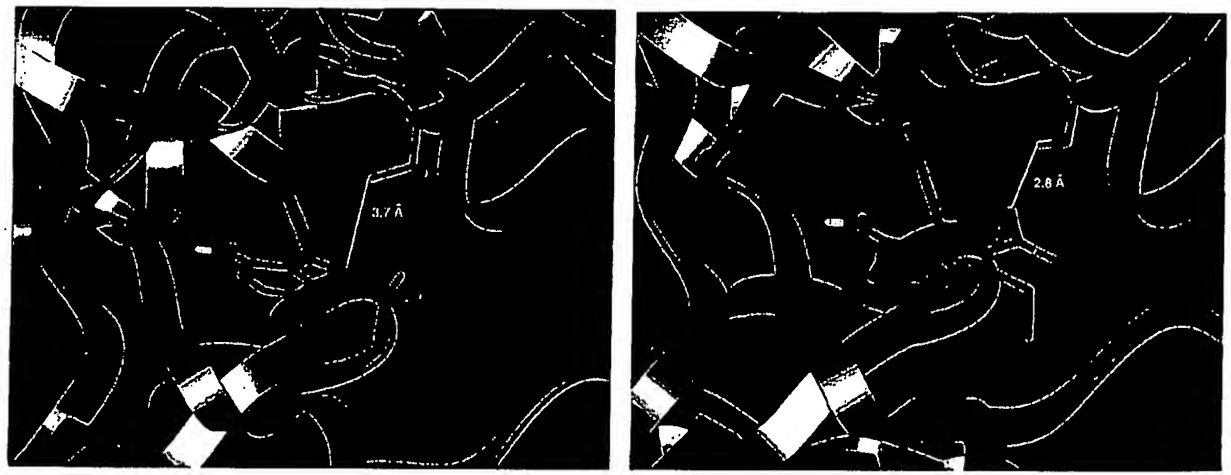
FIG. 19

A



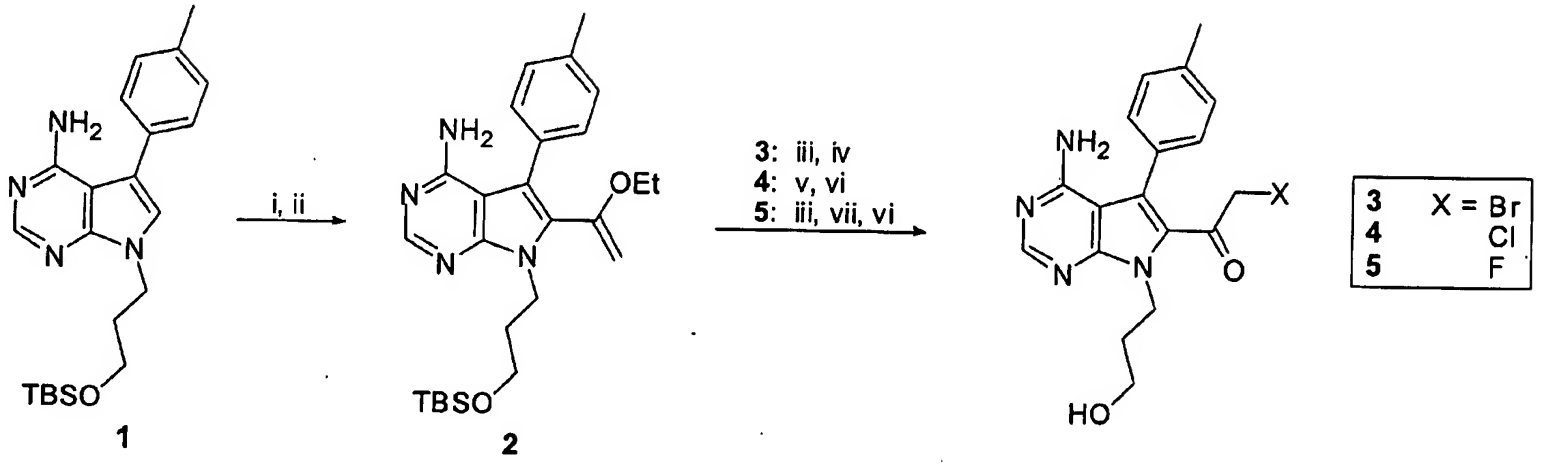
PP1 Designed Electrophile

B



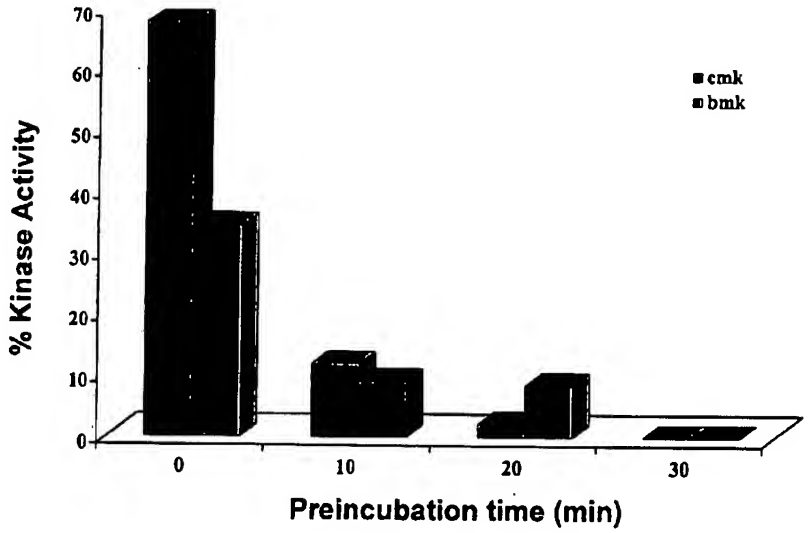
PP1 bound to Hck cmk 4 bound to V282C Hck

FIG. 20



[a]Reagents and conditions: (i) NBS, DMF, rt (ii) $Pd(PPh_3)_4$, α-(ethoxyvinyl)tin, toluene, reflux. (iii) NBS, $NaHCO_3$, H_2O, DMF, -20 °C. (iv) 1:3 1N H Br:THF, 0 °C to rt. (v) NCS, $NaHCO_3$, H_2O, MeCN, rt. (vi) 1:3 1N HCl:THF, 0 °C to rt. (vii) KF, [bmim][BF_4], H_2O, MeCN, 60 °C.

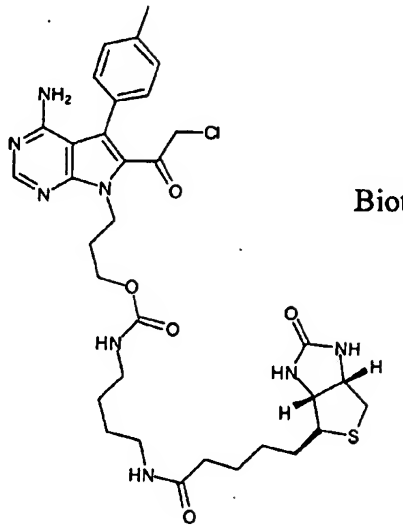
FIG. 21



V282C Fyn was incubated with 10 nM bmk **3** or cmk **4** for varying times. *In vitro* kinase assays were then performed. The graph demonstrates that bmk **3** or cmk **4** exhibit time-dependent inactivation of kinase activity.

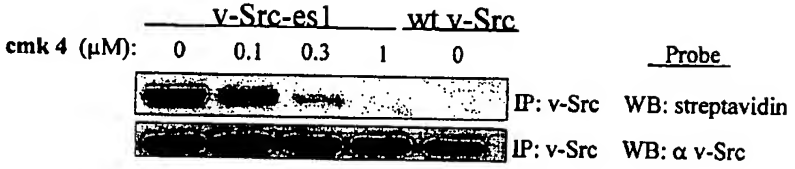
FIG. 22

A



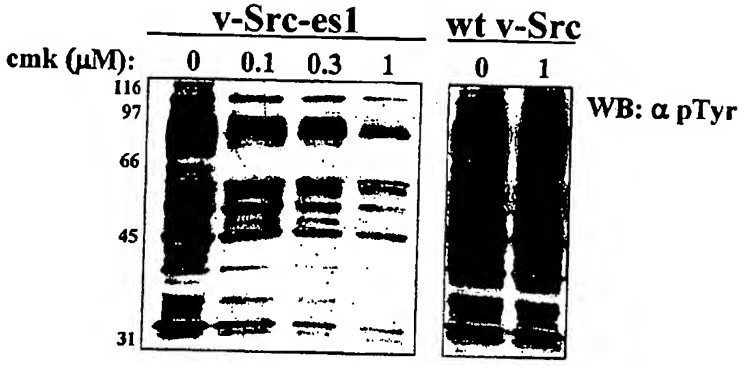
Biotin-cmk

B



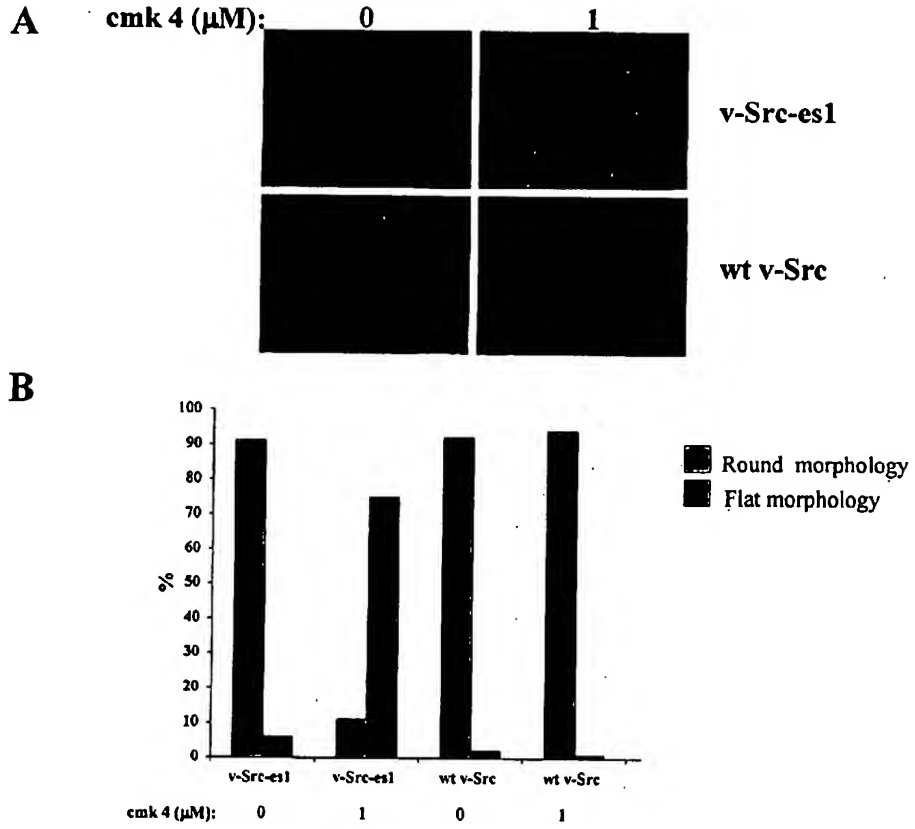
(A) Chemical structure of biotin-cmk. (B) NIH3T3 fibroblasts expressing either v-Src-es1or wt v-Src were pretreated with increasing concentrations of cmk 4 for 2hrs. Whole cell lysates were prepared and treated with biotin-cmk(2 μM) for 1 hr prior to immunoprecipitation of v-Src. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by western blot analysis with anti-v-Src mAb (327) and/or streptavidin-horseradish peroxidase conjugate.

FIG. 23



NIH3T3 fibroblasts expressing either v-Src-es1 or wt v-Src were pretreated with increasing concentrations of cmk 4 for 2hrs. Cells were subsequently washed free of cmk 4 with warmed compound-free medium and incubated for 1 hr. Whole cell lysates were prepared and tyrosine phosphorylated proteins were resolved by SDS-PAGE and analyzed by western blot with anti-phosphotyrosine mAb (4G10).

FIG. 24



(A) Cells expressing either wt v-Src or v-Src-es1 were treated with 1μM cmk 4 for 16 h at 37 °C. Cells were fixed, stained with phalloidin-FITC and visualized by epifluorescence microscopy. (B) 100-200 cells from each treatment condition in (A) for both wt v-Src and v-Src-es1 were categorized as having: flat morphology, round morphology, or inconclusive morphology.

FIG. 25

This Page is inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox